Plant Physiol. (1989) 89, 1024–1027
0032-0889/89/89/1024/04/$01.00/0

Received for publication September 30, 1988
and in revised form October 25, 1988

Communication

Immunochemical Analysis Shows That an ATP/ADP-Translocator Is Associated with the Inner-Envelope Membranes of Amyloplasts from *Acer pseudoplatanus* L.[1]

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ABSTRACT

Pure preparations of intact amyloplasts and chloroplasts, free from mitochondrial contamination, were isolated from cultured cells of the white-wild and green-mutant lines of sycamore (*Acer pseudoplatanus* L.), respectively. A specific rabbit antiserum against yeast mitochondrial cytochrome c_1 only cross-reacted with mitochondrial membranes from the white-wild sycamore cells. The outer and inner envelope-membranes of the two plastid-types were isolated and subsequently analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis to characterize polypeptide patterns in each fraction. Analysis by immunoblotting clearly showed that antiserum against the 29-kilodalton inorganic orthophosphate translocator isolated from pea chloroplasts cross-reacted with a 31-kilodalton polypeptide residing in the inner-envelope membranes from both sycamore chloroplasts and amyloplasts. In contrast, antiserum against the ADP/ATP-translocator isolated from mitochondria of *Neurospora crassa* yielded a positive signal with a 32-kilodalton polypeptide in the inner-membranes isolated from amyloplasts, but not green-mutant chloroplasts. We propose that this 32-kilodalton polypeptide in the amyloplast envelope is a putative ATP/ADP-translocator and its possible functional significance is discussed.

The non-green amyloplast, a uniquely differentiated plastid-type which synthesizes and accumulates starch in the stromal matrix, possesses a unidirectional gluconeogenic pathway in contrast to the vast functional diversity of the chloroplast, including energy (ATP) formation, photosynthetic CO_2-fixation and gluconeogenesis among several others. Based on these reasons, we view the chloroplast and amyloplast each as a "source," "sink," and "gluconeogenic" organelle (16). It can be clearly recognized that although the predominant carbon-flow in chloroplasts is the export of recently fixed carbon assimilates (triose-P) to the cytosol via the Pi-translocator, carbon-partitioning in amyloplasts will be directed to the uptake of carbon-compounds from the cytosol into the stroma and their eventual conversion to starch. In both cases, the Pi-translocator in the inner-envelope membranes is thought to play a crucial role (16), although it remains unclear whether its function in the amyloplasts is truly identical to that operating in the chloroplast envelope. Another key component which possibly resides in the amyloplast inner-envelope is a putative ATP/ADP-translocator (16); since this plastid type lacks the energy-producing machinery associated with the thylakoid membranes in chloroplasts, the ATP which is required for the progress of gluconeogenesis (*i.e.* ADP-glucose formation) must be imported from the cytosol.

To address the question of whether or not an ATP/ADP-translocator is specifically present in the inner membrane of the amyloplast envelope, we have exploited immunoblot analysis using antiserum against the ADP/ATP-translocator isolated from the mitochondrial membranes of *Neurospora crassa* (22). Throughout this investigation related to the biochemistry of the amyloplasts, we have used functional chloroplasts isolated from the green-mutant cell-line of sycamore as a critical control system (16, 17).

MATERIALS AND METHODS

Culture methods and the isolation of intact amyloplasts and chloroplasts from white-wild and green-mutant cells of sycamore (*Acer pseudoplatanus* L.), respectively, were exactly the same as those reported previously (16, 17). In addition, the specific mitochondrial marker-enzyme Cyt c_1 has been examined in the plastid preparations as well. Mitochondria were isolated from the white-wild cells according to the method of Ali *et al.* (1).

The method for separating the outer- and inner-envelope membranes from isolated intact amyloplasts and chloroplasts by the protocol originally developed by Cline *et al.* (3) has been reported previously (8, 9). The specific localization of Mg^{2+}-ATPase in the inner and acyl-CoA synthetase in the outer-envelope membranes of plastids has been described in the previous paper (8, 9). Total mitochondrial membranes were isolated by homogenizing intact mitochondria from white-wild cells in a hypotonic solution containing 10 mM

[1] This is Paper No. 79 in the series "Structure and Function of Chloroplast Proteins." This research was supported in part by research grants from the Ministry of Education, Science and Culture (Mombusho) of Japan and the Nissan Science Foundation (Tokyo).

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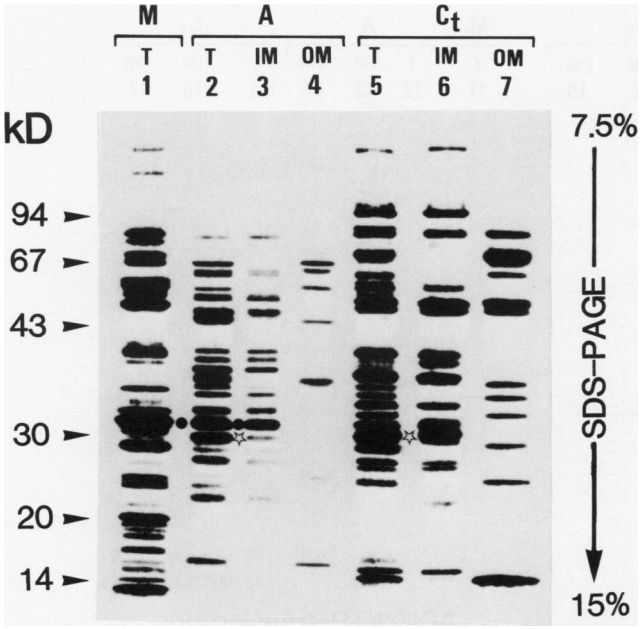

Figure 1. SDS-PAGE analysis of polypeptides from membrane fractions obtained from mitochondria (M), amyloplasts (A) and chloroplasts (C_t) of sycamore cells. For A and C_t, in addition to the total envelope membranes (T), the inner (IM) and outer (OM) membranes were electrophoresed. A 10-μg protein sample was loaded in each lane. Lane 1, total membranes of mitochondria from white-wild cells; lanes 2 to 4, total, inner, and outer envelope membranes of amyloplasts; lanes 5 to 7, total, inner, and outer envelope membranes of chloroplasts, respectively. The 31- and 32-kD polypeptides are marked (☆) and (●), respectively.

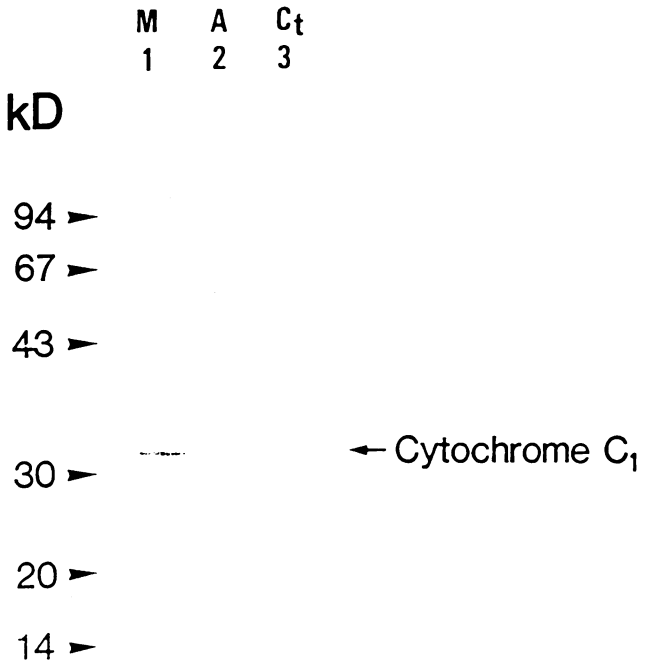

Figure 2. Western blot analysis of mitochondrial membranes, and amyloplast and chloroplast envelope-membranes using antiserum against yeast Cyt c_1. Samples (150 μg protein) of total membranes from white-wild mitochondria (M, lane 1) and amyloplast (A, lane 2) and chloroplast (C_t, lane 3) envelopes were subjected to SDS-PAGE, followed by immunoblotting using alkaline phosphatase-conjugated goat anti-rabbit antiserum (IgG) raised against yeast Cyt c_1.

Mops-NaOH (pH 7.8), 1 mM $MgCl_2$, and 1 mM EDTA with a ground-glass homogenizer. The resulting total membrane fraction was then sedimented at 200,000*g* for 30 min, at 4°C.

The characterization of polypeptide profiles of the total mitochondrial membranes and the chloroplast and amyloplast total envelope fractions, as well as those from the separated outer and inner plastid envelope membranes, was performed using SDS (1.5%)-polyacrylamide gradient gel (7.5 to 15%) electrophoresis. In order to improve resolution and sensitivity, a 12-cm long slab-gel was employed, together with silver staining (18).

Immunoblot analysis was performed using alkaline phosphatase-conjugated goat anti-rabbit IgG[3] following the procedure of Blake *et al.* (2). The antisera used in this investigation were kindly provided by Dr. T. Hase (Nagoya University) for anti-yeast Cyt c_1 IgG (22); Dr. U. Flügge (University of Göttingen, FRG) for anti-pea chloroplast Pi-translocator IgG (5, 6); and Dr. W. Neupert (University of München, FRG) for *Neurospora crassa* ADP/ATP-translocator IgG (22). Protein content was determined by a modified Lowry method using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

The polypeptide profiles of the total mitochondrial membranes and the amyloplast and chloroplast envelope-membranes resolved by SDS-PAGE are presented in Figure 1. It is clear that mitochondrial membrane proteins are distinguishable from the envelopes of the two plastid-types. We are particularly interested in the polypeptide patterns of the inner membranes isolated from the amyloplast and chloroplast envelopes. The profiles visualized by silver staining are quite different between them (lanes 3 and 6), and densitometric tracing clearly indicates that there are specific bands characteristic of each inner-membrane fraction (data not shown). It should be noted that, consistent with our previous reports (8, 9, 16), there is a major polypeptide (32-kD) (closed circle) in the amyloplast inner-membrane which is clearly separable from a faint band of 31-kD band corresponding to the Pi-translocator (see below and Fig. 3) (star), whereas the 31-kD protein is a dominant molecular species in the inner-membrane of the chloroplasts.

Before proceeding to the next step of immunoblot analysis, the absence of mitochondrial membrane contamination in the two plastid-type envelope-membrane preparations must be rigorously established. For this purpose, the total membrane fractions derived from mitochondria and the amyloplast and chloroplast envelopes were treated with antiserum against yeast Cyt c_1 and blotting data are shown in Fig. 2. While the mitochondrial membranes gave a strong positive signal for this 32-kD polypeptide (lane 1), neither the amyloplast (lane 2) nor chloroplast (lane 3) envelope-membranes contained a cross-reactive polypeptide.

The results of the most important immunoblotting experiments are presented in Figure 3. While the mitochondrial

[3] Abbreviation: IgG, immunoglobulin G.

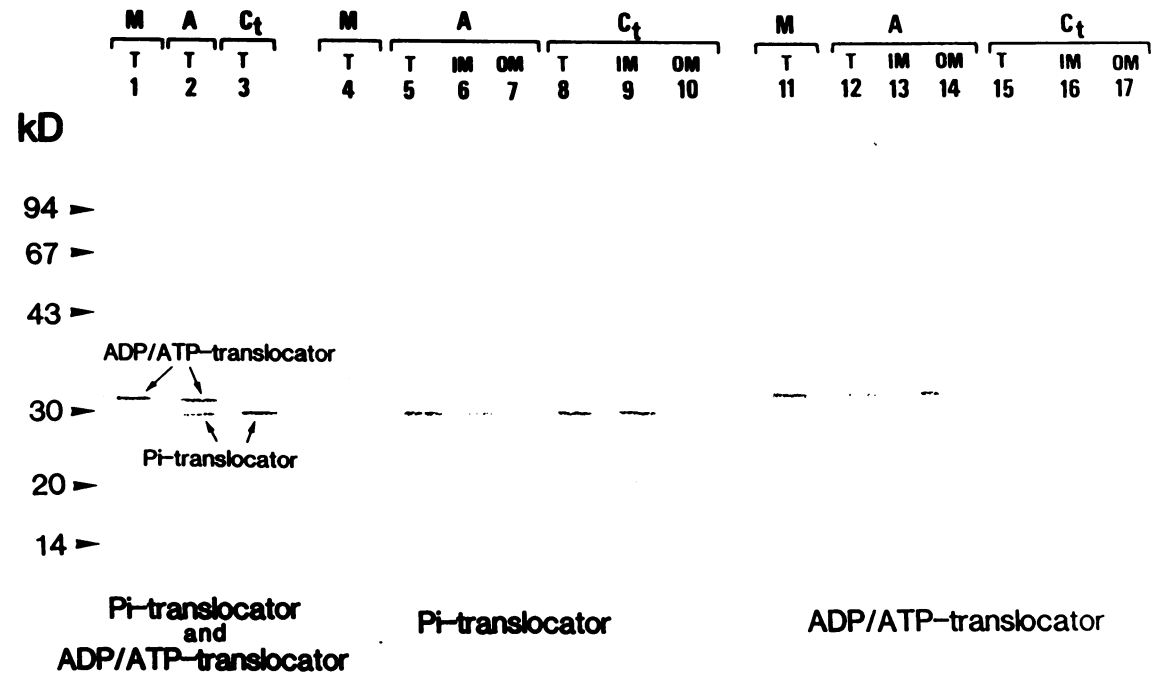

Figure 3. Western blot analysis of mitochondrial, amyloplast and chloroplast membrane fractions using antisera against the Pi-translocator (pea chloroplast) and ADP/ATP-translocator (*N. crassa*). The basic procedures employed were the same as those described in Figures 1 and 2, and 150 μg protein were loaded per lane. Membrane fractions of total (T), inner (IM) and outer (OM) envelopes were subjected to SDS-PAGE and subsequently charged with antisera against the Pi-translocator and ADP/ATP-translocator (lanes 1–3) or the Pi-translocator (lanes 4–10) or ADP/ATP-translocator (lanes 11–17), separately.

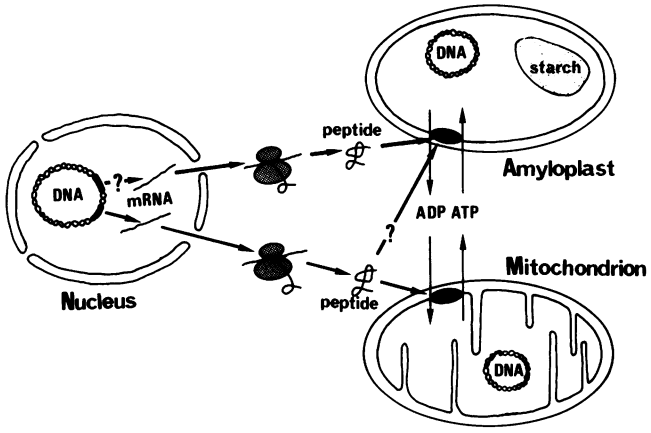

Figure 4. Schematic representation of the possible expression of the nuclear gene encoding the adenylate-translocator and its targeting into different organelles, *i.e.* mitochondria and amyloplasts. The identical polypeptide may be encoded by the same member(s) of a nuclear multigene family, then targeted to the ADP/ATP-translocator in the mitochondria and ATP/ADP-translocator in the amyloplast. On the other hand, the polypeptide targeted to the amyloplast may be encoded by a different gene-member.

(lane 1) and chloroplast envelope (lane 3) total membranes cross-react only with the antisera against the ADP/ATP-translocator (*N. crassa*) or Pi-translocator (pea), respectively, the amyloplast total envelope membranes react with both (lane 2). Although the inner-membranes of both amyloplasts and chloroplasts specifically contain the 31-kD Pi-translocator polypeptide (lanes 6 and 9), the antiserum against the mitochondrial ADP/ATP-translocator only cross-reacted with the amyloplast inner-membranes (lane 13) and neither the inner chloroplast envelope (lane 16) nor the outer-membranes of both plastid envelopes (lanes 14 and 17). The molecular size of this antigenically positive polypeptide in the amyloplast inner-envelope membrane is about 32-kD, similar to its mitochondrial counterpart (lane 11).

The above results indicate the presence of the Pi-translocator and ADP/ATP-translocator associated with the amyloplast inner-envelope membrane. The importance of these two polypeptides in the carbon-partitioning of this "gluconeogenic" plastid-type has been discussed in our previous paper (16), and their structural and functional nature must be more critically examined by future investigations. In amyloplasts, the import of ATP is absolutely necessary for various important metabolic purposes, including the synthesis of ADP-glucose and the transmembrane import of nuclear-encoded preproteins which are engaged in starch biosynthesis in the stroma. Since the direction of ATP transport across the amyloplast envelope-membrane is the reverse of that in mitochondria, we propose the translocator engaged in this process as an ATP/ADP-translocator. In this context it should be noted that Liedvogel and Kleinig (14) reported the presence of an adenylate translocator in chromoplast membranes isolated from the daffodil flower, which is presumably involved in fatty acid biosynthesis. On the other hand, it has been reported that the ATP-transport activity of the isolated C_3-chloroplast is generally low (11–13), although it depends on the developmental stages of the chloroplasts (21) and kinetics of chloroplast-envelope transport can vary during chloroplast development (7).

Since ADP/ATP translocator is a nuclear-encoded protein which is the abundant mitochondrial membrane component (22), a pertinent question to be answered is as schematically illustrated in Figure 4, whether or not the same nuclear gene encodes the identical polypeptide for these two different organelle membranes (mitochondria and amyloplasts) or if two different structural genes exist. In addition to these two unresolved mechanistic issues, the processing of the amyloplast ATP/ADP-translocator should also be studied, since the mitochondrial translocator is transported in the form of a precursor protein having the same molecular size as that of the mature form (4, 15, 20, 23). Another interesting possibility is that the modification of the same transcript results in two different end products as in the case of yeast invertase (19) (see Fig. 4).

ACKNOWLEDGEMENTS

The authors deeply thank Dr. R. Chollet for critical reading of the manuscript. They are also grateful to Dr. T. Hase (Nagoya University) for anti-yeast Cyt c_1 IgG, Dr. U. Flugge (University of Göttingen, FRG) for anti-pea chloroplast Pi-translocator IgG and Dr. W. Neupert (University of München, FRG) for anti-*Neurospora crassa* ADP/ATP-translocator IgG. J. N. wishes to express her sincere thanks to the Hitachi Foundation for providing her with a predoctoral student scholarship.

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