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Import and processing of proteins by castor bean leucoplasts

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The precursor of plastocyanin, a thylakoid lumen protein, is imported by castor bean leucoplasts and processed to an intermediate form by a stromal processing peptidase of similar properties and specificity to the corresponding chloroplast enzyme. No further maturation is observed, indicating that the internal protein transport and maturation system present in the chloroplast is absent in leucoplasts.

Chloroplast; Leucoplast; Protein transport; Processing; Thylakoid lumen

1. INTRODUCTION

Several features of the synthesis of chloroplast proteins are now well established. Most chloroplast proteins are synthesised in the cytosol as precursors containing aminoterminal presequences [1] and are imported post-transitionally into the chloroplast by an ATP-dependent mechanism [2]. On import, precursors of stromal proteins are processed to their mature sizes by a soluble stromal processing protease [SPP] [3] whereas precursors of thylakoid lumen proteins are processed, probably by the same protease, to intermediate size forms. These intermediates are subsequently imported into the thylakoid lumen and processed to the mature size by a second peptidase located in the thylakoid membrane [4–6].

Other types of plastid are also capable of protein import. The abundant leucoplasts of castor bean endosperm are non-photosynthetic and function in the synthesis of fatty acids. They lack an organised internal membrane system and are deficient in most of the enzymes involved in photosynthesis and starch synthesis [7,8]. They do, however, contain the enzyme ribulose bisphosphate carboxylase (RUBISCO) and isolated leucoplasts have been shown to import the precursor of the pea chloroplast small subunit [9], indicating that the basic import machinery is probably common to both plastid types.

In this report we have assessed whether other components of the chloroplast import machinery have functional counterparts in the leucoplast. We show that the leucoplast contains an apparently identical stromal processing activity but we find no evidence of an internal protein-translocating membrane system.

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2. MATERIALS AND METHODS

2.1. *Isolation of plastids*

Castor bean seeds (*Ricinis communis*) were soaked for 24 h and germinated in moist vermiculite at 30°C in the dark for 3–4 days. Testa and cotyledons were removed and the endosperm from 100 beans was chopped in homogenization buffer (12% w/v sucrose; 1 mM EDTA; 1 mM $MgCl_2$; 10 mM KCl; 150 mM Tricine-KOH, pH 8.0, 100 mM lactose, 1 mM PMSF). The homogenate was filtered through 4 layers of muslin and centrifuged at 270×g for 7 min. The pellet was resuspended in a small volume of homogenization buffer and layered onto sucrose gradients composed of 1 ml 60% sucrose cushion, 9 ml continuous 30–60% sucrose gradient overlayed with 1 ml 30% sucrose. The gradients were centrifuged at 40 000 rpm for 1 h (SW40, Beckman L-8). In initial experiments the identity and purity of the plastid band was confirmed by fractionating one gradient into 0.5 ml aliquots and assaying each aliquot for the marker enzymes fumarase (mitochondria), isocitrate lyase (glyoxysomes) and RUBISCO (plastids). In subsequent experiments the plastid bands were pooled, diluted gradually in 5 vols SRM (50 mM Hepes-KOH; 300 mM sorbitol pH 8.0) and pelleted at 10 000×g for 10 min.

2.2. *Assay of leucoplast stromal processing peptidase*

Pelleted leucoplasts were lysed in 500 μl 20 mM Tris-HCl, pH 8.0 at 4°C for 20 min, followed by centrifugation at 10 000×g for 10 min to generate a stromal supernatant. Precursors of *Silene pratensis* plastocyanin and ferredoxin were generated by transcriptions of cDNAs [10,11] using SP6 RNA polymerase followed by translation in a wheat-germ system in the presence of [^{35}S]methionine [5]. In processing assays, 20 μl stromal extract or chloroplast SPP and 1 μl translation product were incubated at 27°C for 60 min, after which samples were analysed by SDS-PAGE and fluorography. Pea chloroplast SPP was prepared as described [3].

2.3. *Import assays*

Castor bean leucoplasts pelleted as above were resuspended in 200 μl SRM. Each import assay (40 μl) contained 25 μl leucoplasts, 5 μl pre-plastocyanin translation product, 10 mM $MgCl_2$, 10 mM ATP, and 20 mM methionine. Incubation was at 25°C for 45 min, after which appropriate samples (see figure legend) were incubated with trypsin (150 μg ml^{-1}) for 30 min at 4°C, followed by treatment with 2 mM PMSF. The import mixtures were then diluted with 5 ml SRM, centrifuged, and lysed as above to generate stromal and membrane fractions.

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3. RESULTS AND DISCUSSION

Leucoplasts were isolated from germinating castor beans by sucrose density ultracentrifugation when RUBISCO activity was maximal (3–4 days germination). Fractions removed from the gradients were assayed for marker enzyme activities and the results are shown in fig.1. The two major protein peaks coincide with the activities of fumarase (mitochondria) and RUBISCO (plastids). Some isocitrate lyase (glyoxysomes) activity was detected in the denser fractions of the plastid peak. For subsequent experiments, therefore, only the very early plastid fractions (11–13), devoid of isocitrate lyase activity, were used.

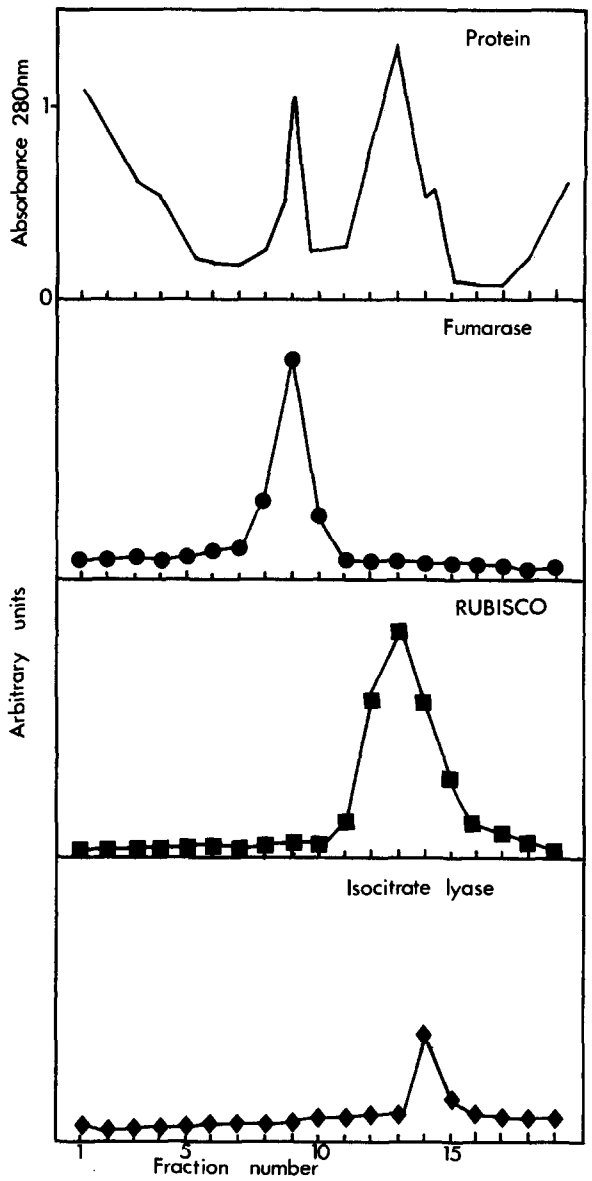

Fig.1. Sucrose density gradient fractionation of castor bean organelles. Germinating castor bean seeds were homogenised and fractionated as described in section 2. Fractions were measured for protein concentration and assayed for fumarase, RUBISCO and isocitrate lyase activity as described [8,9]. Fraction 1 = top of gradient.

A stromal extract was prepared from the plastids and compared with partially purified pea stromal processing peptidase [SPP] for the ability to process precursors to the stromal protein ferredoxin, [pFD] and the thylakoid lumen protein plastocyanin [pPC]. Fig.2A shows that the leucoplast extract is capable of processing pFD (lane 2) to a lower molecular weight product (lane 4) that migrates with identical mobility to the processed product generated by SPP shown in lane 3. Both processed products comigrate with mature ferredoxin produced by import of pFD into isolated pea chloroplasts (lane 1). Similarly, when pPC (figure 2B lane 2) is incubated with the leucoplast extract a processed product is generated that is identical in size to that produced by SPP (compare lanes 3 and 4). This product is larger than mature plastocyanin but co-migrates with the plastocyanin stromal intermediate produced during import of pPC into chloroplasts in vitro (lane 1). These data indicated that castor bean leucoplasts contain a stromal protease of similar specificity to pea SPP. The two proteases also show similar sensitivities to protease inhibitors (not shown).

To investigate the leucoplast import capabilities pPC was incubated with isolated leucoplasts under conditions similar to those routinely used for chloroplast import experiments. After import the leucoplasts were fractionated into stromal and membrane components.

In the presence of added ATP the leucoplasts import pPC and process it to the stromal intermediate form (fig.3, compare lane 4 with pPC, lane 1 and pPC import markers, lane 8). Some unimported pPC can be seen bound to the membrane fraction (lane 5). In the absence of ATP, pPC import is inhibited (compare lanes 4 and 2) but precursor binding to the membrane is not (lane

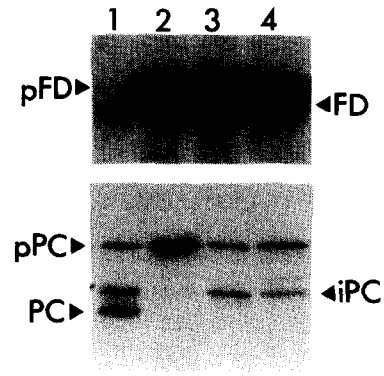

Fig.2. Assay of stromal processing peptidases from leucoplasts and chloroplasts. (A) pFD (lane 2) was incubated with leucoplast stroma (lane 3) or partially purified chloroplast SPP (lane 4) as detailed in section 2. Lane 1, marker for mature-size FD. (B) As in A but using pPC as a substrate. Markers for intermediate and mature-size PC (lane 1) were prepared as described [5]. PC, iPC, mature and intermediate forms of plastocyanin; FD, mature-size ferredoxin.

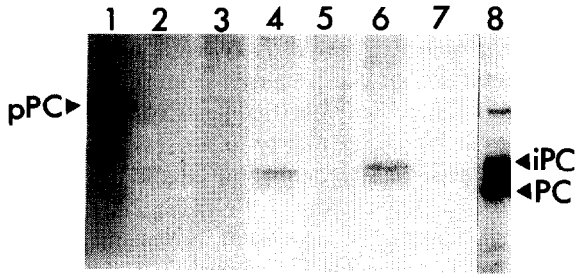

Fig.3. Import of pPC into leucoplasts. pPC (lane 1) was incubated with leucoplasts in the absence of added ATP (lanes 2 and 3) or in the presence of 10 mM ATP (lanes 4–7). After import, one mixture was trypsin-treated (lanes 6 and 7). Samples were then fractionated into stromal (lanes 2,4,6) or membrane (lanes 3,5,7) fractions. Lane 8, PC import marker as in fig.2. Symbols as in fig.2.

3). The small amount of pPC imported in lane 2 reflects the presence of some ATP contributed by the wheat germ lysate. Confirmation of the internalization of the pPC intermediate within the leucoplast is demonstrated by its protection to protease added prior to plastid lysis and fractionation (lane 6) while the pPC bound to the outside cytoplasmic face of the membrane is completely susceptible to proteolysis (lane 7). Certain features of leucoplast and chloroplast protein transport appear, therefore, to be identical: binding of precursors to the plastid membrane, ATP-dependent import, and processing by a stromal processing peptidase to the mature size (stromal proteins) or to intermediate forms (thylakoid lumen proteins). In one important aspect, however, the two plastid types differ significantly: most imported pPC is converted to the mature size by chloroplasts [4,5] whereas no mature size PC is produced by the leucoplasts; maturation proceeds only to the stromal intermediate stage, suggesting that leucoplasts do not contain an internal protein transport system which is capable of recognizing the plastocyanin processing intermediate. This finding contrasts with a previous report [12] in which complete maturation of this precursor was observed in non-photosynthetic chromoplasts and root plastids of transgenic tomato. Possibly, these plastids differ from the leucoplasts used in the present study by containing small quantities of an internal membrane system with similar protein import/maturation properties to the thylakoid system.

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