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Specific transport of inorganic phosphate, glucose 6-phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate into amyloplasts from pea roots

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Evidence is provided that amyloplasts from pea roots contain a translocator which transports, in a counter exchange mode, phosphate, glucose 6-phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate. The translocator has a low affinity for 2-phosphoglycerate and glucose 1-phosphate. Metabolite transport was measured by silicone oil filtering centrifugation either directly by uptake of radioactive labelled compounds or indirectly by back exchange.

Phosphate transport; Glucose 6-phosphate; Triose phosphate; Phosphoglycerate, 3-; Amyloplast envelope; (*Pisum sativum*)

1. INTRODUCTION

Leaf chloroplasts have a phosphate translocator catalyzing the transport of triose phosphates and 3-PGA in exchange for P_i [1]. Hexose phosphates are not transported across the chloroplast envelope. The question arose whether this translocator is also operative in amyloplasts. Studies with amyloplasts from soybean, cauliflower buds and maize kernels indicated that this is probably the case [2–4]. On the other hand, investigations with amyloplasts from other tissues (e.g. wheat endosperm) showed that Glc1P and/or Glc6P could be incorporated into starch [5–7] and it was found with pea root plastids that nitrite reduction can be supported by Glc6P [8]. These results suggested that amyloplasts, in contrast to chloroplasts, are able to transport hexose phosphates. The measurement of metabolite transport into amyloplasts turned out to be extremely difficult. Emes and Traska [9] found an uptake of [^{32}P]phosphate into pea root amyloplasts, which was reduced in the presence of triose P or 3-PGA. The measurements did not allow the evaluation of the corresponding kinetic constants. Whilst these results were not inconsistent with there being a phosphate translocator associated with the amyloplasts, the above mentioned authors themselves stated that such an interpretation was still equivocal.

The studies described here aim to investigate the specificity of the transport of phosphorylated intermediates into amyloplasts. Evidence will be presented that these plastids possess a phosphate translocator transporting in a counter exchange mode not only P_i, triose P and 3-PGA, but also Glc6P.

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Abbreviations: P_i, inorganic phosphate; Glc6P, glucose 6-phosphate; Glc1P, glucose 1-phosphate; DHAP, dihydroxyacetone phosphate; 3-PGA, 3-phosphoglycerate; 2-PGA, 2-phosphoglycerate; triose P, triose phosphate; PLP, pyridoxal 5′ phosphate; 6 PGDH, 6-phosphogluconate dehydrogenase; GOGAT, NADH-glutamate-synthase

2. MATERIALS AND METHODS

2.1. *Preparation of amyloplasts*

Peas (*Pisum sativum* L., var. Kleine Rheinländerin, van

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Waveren, Göttingen-Rosdorf) were surface-sterilized, soaked overnight in water and germinated on moistened tissue paper at 27°C in the dark for 4 days. After 3 days enzymes of the nitrogen and carbohydrate metabolism were induced by incubation in 20 mM KNO_3 for 20 min. The preparation of amyloplasts was based on the methods of Emes and England [10] and Oji et al. [11]. 200 g roots were cut into pieces in 200 ml of a medium containing 50 mM Tricine-KOH (pH 7.9), 0.33 M sorbitol, 1 mM EDTA, 1 mM $MgCl_2$ and 0.1% (w/v) BSA (medium A) and gently homogenated with a Polytron homogenizer at 4°C. The homogenate was squeezed through two layers of miracloth/four layers of cheesecloth, one layer of 40 μm nylon net/two layers of cheesecloth and one layer of 7 μm nylon net. Cell debris and free starch grains were sedimented by centrifugation (200 × g, 3 min). Portions of the supernatant (20–25 ml) were underlaid with 10 ml of a medium containing 50 mM Tricine-KOH (pH 7.9), 0.33 M sorbitol, 0.1% (w/v) BSA and 10% (v/v) Percoll and centrifuged (6 min, 4000 × g). The sediments were resuspended in medium A without BSA (medium B).

The amyloplast preparation was essentially free of other contaminating organelles. The activities of marker enzymes recovered in the plastid fraction as % of the supernatant from the first centrifugation step were as follows. Amyloplast marker: nitrite reductase 30%, alkaline inorganic pyrophosphatase 30%, 6 PGDH 10%; cytosol marker: alcohol dehydrogenase 0.8%; mitochondrial marker: citrate synthase 4%; vacuolar marker: α-mannosidase 4%; peroxisomal marker: hydroxypyruvate reductase 0.8%; endoplasmic reticulum: cytochrome-c reductase 3%. From latency measurements of GOGAT and 6 PGDH, the plastids were about 90% intact. Electron microscopy revealed an average diameter of the plastids between 2 and 3 μm and that both envelope membranes were retained. The plastids contained starch grains, an internal membrane system and plastoglobuli.

2.2. *Enzyme assays and protein determination*

If not mentioned otherwise the measurements were carried out at 25°C in a final volume of 700 μl. The activities of the following marker enzymes were assayed as described: 6 PGDH [3]; GOGAT [10]; citrate synthase [12]; alcohol dehydrogenase [13]; alkaline inorganic pyrophosphatase [14] (37°C); α-mannosidase [15] (37°C); nitrite reductase [16] (30°C); antimycin-A-insensitive cytochrome-c reductase [17]; hydroxypyruvate reductase [18]. For the determination of enzyme latency and plastid intactness, the enzyme activities of GOGAT and 6 PGDH were measured in the presence and absence of 0.33 M sorbitol. Alternatively the latency could be measured by rupture of the plastid envelope membranes with 0.1% Triton X-100. Protein concentration was measured according to Lowry.

2.3. *Transport measurements*

The silicone oil filtering centrifugation was carried out according to Heldt and Sauer [19]. 200 μl amyloplasts containing a latent 6 PGDH activity of 25–35 nmol/min were layered over 20 μl of 10% $HClO_4$ and 70 μl silicone oil (AR 200), followed by 10 s incubation (unless otherwise stated) at 20°C with substrate. The substrate uptake was terminated either by inhibitor stop using a PLP concentration (20 mM), which was high enough to inhibit the substrate uptake immediately, and centrifugation for 120 s or only by centrifugation for 120 s. The density of the silicone oil mixture used was adjusted in such a way that after centrifugation about 90% of the amyloplast marker enzyme GOGAT appeared in the sediment. Correction for external medium adhering to the plastid or present in the intermembrane space between the two envelope membranes was done in order to calculate the amyloplast volume. The sorbitol-impermeable space amounted to 39 ± 11% of the total $[^3H]H_2O$-permeable space in a medium containing 0.33 M sorbitol. For measurements of back exchange the pellets of the 4000 × g centrifugation (see section 2.1.) were resuspended in medium B, preincubated with 2 mM $[^{32}P]$phosphate (20 min, 4°C) and washed twice with medium B (4000 × g, 2 min). The silicone oil filtering centrifugation had been done as described, with the exception, that unlabelled substrates were added. Radiochemicals were purchased from Amersham. $[^{14}C]$Glc6P was prepared using glucose, ATP and hexokinase. The reaction was stopped after complete consumption of glucose (95°C, 3 min). $[^{14}C]$Glc6P was separated from residual ATP by thin layer chromatography (cellulose plate; solvent: $MeOH/NH_3$ (25%)/H_2O = 7:1:2).

3. RESULTS AND DISCUSSION

3.1. *Metabolite uptake into amyloplasts*

In the experiments of fig.1 the time course for the uptake of P_i and Glc6P was measured. The uptake of inorganic phosphate and Glc6P into the sorbitol-impermeable $[^3H]H_2O$-space was terminated by inhibitor stop using PLP, followed by centrifugation 5 s later. In control experiments, PLP and the labelled substrates were added together and centrifuged after 5 s. The uptake was determined from the difference of the two experiments. For both P_i and Glc6P a defined uptake kinetic is obtained. After an initial linear phase of about 10 s the uptake slows down and finally reaches a plateau, leading to an accumulation of both substrates.

Fig.2 shows the concentration dependence of the phosphate uptake. A double reciprocal plot of the data yields a linear function, indicating that the uptake has a saturation characteristic. These measurements were also carried out without inhibitor stop. Without inhibitor stop the uptake rate is overestimated, but nevertheless the K_m value thus obtained is unaltered. For simplification of the measurements the uptake experiments dealt with hereafter were performed without inhibitor stop. The specificity of the P_i and the Glc6P uptake was investigated in the experiments of figs 3 and 4. The transport of both substances was competitively inhibited by each other and by DHAP. It

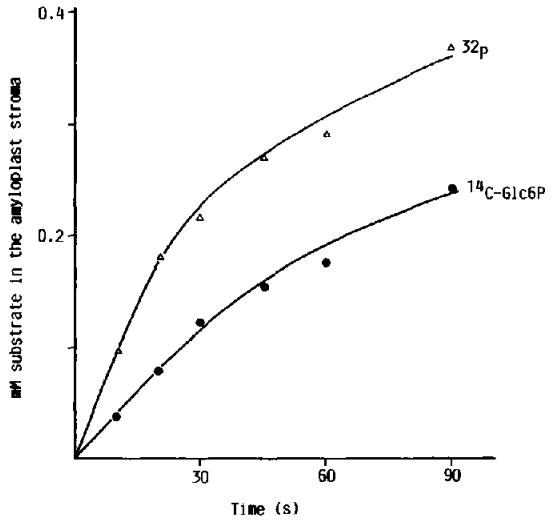

Fig.1. Uptake of [^{32}P]phosphate (0.1 mM) and [^{14}C]Glc6P (0.1 mM) into pea root amyloplasts as terminated by inhibitor stop. Control values: P_i uptake 0.05 mM; Glc6P uptake 0.03 mM.

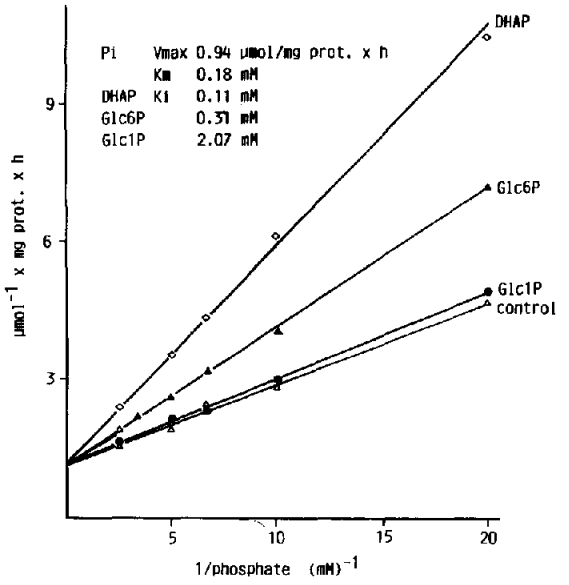

Fig.3. Double reciprocal plot of the concentration dependence of the [^{32}P]phosphate uptake into pea root amyloplasts without and with competing substrates (0.2 mM).

may be noted that Glc1P had only a low inhibitory effect on transport of both P_i and Glc6P.

3.2. *Measurements of metabolite transport by back exchange*

The kinetic constants of metabolite uptake can be measured directly by the uptake of radioactive labelled substances or indirectly by back exchange. To prove our assumption that DHAP, P_i and Glc6P are transported by the same carrier, amyloplasts were preloaded with [^{32}P]phosphate and the concentration dependence of the uptake of

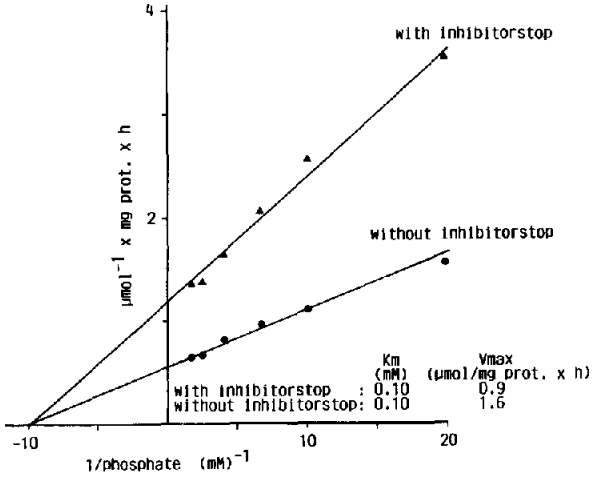

Fig.2. Double reciprocal plot of phosphate uptake into pea root amyloplasts: measurement with and without inhibitor stop.

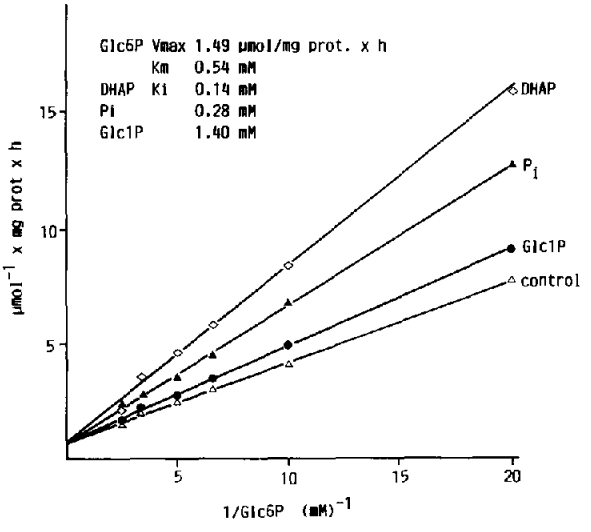

Fig.4. Double reciprocal plot of the concentration dependence of the [^{14}C]Glc6P uptake into pea root amyloplasts without and with competing substrates (0.2 mM).

Table 1

Specificity of the phosphate translocator in amyloplasts from pea roots as determined by back exchange (n = number of measurements)

Substrate	K_m (mM ± SD)	V_{max} (μmol/mg prot × h ± SD)	n
P_i	0.06 ± 0.01	0.76 ± 0.14	4
DHAP	0.04 ± 0.01	0.87 ± 0.11	4
3-PGA	0.10 ± 0.04	0.67 ± 0.14	4
Glc6P	0.11 ± 0.02	0.57 ± 0.11	4
Glc1P	not measurable		2
2-PGA	not measurable		2

unlabelled substances was determined by measuring the release of [^{32}P]phosphate. As shown in table 1 there is a counter exchange of DHAP, 3-PGA and Glc6P, whereas 2-PGA and Glc1P do not produce any measurable release of P_i. Although the K_m values for the back exchange are lower than the K_m or K_i values obtained in P_i or Glc6P uptake experiments, the relationship between the values for the different substrates, e.g. K_m P_i/K_m Glc6P in the uptake and the back exchange experiments is very similar.

It appears from our data that the amyloplast envelope contains a phosphate translocator which is similar to the known chloroplast translocator [1] in transporting P_i, DHAP and 3-PGA in a counter exchange mode, but which differs in transporting also Glc6P. The transport of Glc6P is rather specific, Glc1P shows only a weak interaction with the translocator. Our results demonstrated that in a single plant species the phosphate translocators of the chloroplasts and the root plastids are distinctly different in their transport specificity. This seems to be a reflection of the different functions of these plastids. Whereas the main function of the chloroplasts is the provision of fixed carbon to the cell in the form of triose P, the major function of the root plastids is the reduction of nitrite [8], for which the redox equivalents are supported by oxidative pentose phosphate pathway located in the plastid stroma. Since plastids from different tissues do not appear to contain fructose bisphosphatase activity ([6]; own results, unpublished), the operation of the oxidative pentose phosphate pathway in these plastids would require an uptake of Glc6P and release of triose P. Synthesis of starch would involve the uptake of Glc6P and the release of P_i. The specificity of the root plastid phosphate translocator described above suits both functions. The question arises as to what extent the structures of the plastid phosphate translocators in leaf and root cells are different. The cDNA structure for the chloroplast phosphate translocator has been recently elucidated [20]. A comparison of the structure of the root plastid translocator may provide an insight into the mechanism of plastid metabolite transport.

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