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Phosphate Translocator of Isolated Guard-Cell Chloroplasts from *Pisum sativum* L. Transports Glucose-6-Phosphate[1]

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Chloroplasts were isolated from ruptured guard-cell protoplasts of the Argenteum mutant of *Pisum sativum* L. and purified by centrifugation through a Percoll layer. The combined volume of the intact plastids and the uptake of phosphate were determined by silicone oil-filtering centrifugation, using tritiated water and [^{14}C]sorbitol as membrane-permeating and nonpermeating markers and [^{32}P]phosphate as tracer for phosphate. The affinities of the phosphate translocator for organic phosphates were assessed by competition with inorganic phosphate. The affinities for dihydroxyacetone phosphate, 3-phosphoglycerate (PGA), and phospho*enol*pyruvate were in the same order as those reported for mesophyll chloroplasts of several species. However, the guard-cell phosphate translocator had an affinity for glucose-6-phosphate that was as high as that for PGA. Guard-cell chloroplasts share this property with amyloplasts from the root of pea (H.W. Heldt, U.I. Flügge, S. Borchert [1991] Plant Physiol 95: 341–343). An ability to import glucose-6-phosphate enables guard-cell chloroplasts to synthesize starch despite the reported absence of a fructose-1,6-bisphosphatase activity in the plastids, which would be required if only C_3 phosphates could enter through the translocator.

Guard cells possess chloroplasts (there are exceptions: Nelson and Mayo, 1975). They manifest linear photosynthetic electron transport (Zeiger et al., 1981; Outlaw et al., 1981), yet their Rubisco activity is extremely low. Whereas in *Pisum sativum* the Chl content of guard cells (approximately 0.9 pg) corresponds to about 1/80 that of mesophyll cells, their Rubisco activity (0.12 pmol cell^{-1} h^{-1}) is just about 1/300 that of an ordinary mesophyll cell (Reckmann et al., 1990). The rate of production of hexoses through the photosynthetic pentose-phosphate cycle in guard cells could not deliver more than 2% of the flow of osmotica required for stomatal opening (Reckmann et al., 1990). The major stream of solutes must come from an import of K^+ into the guard cells and from the production of malate within them.

Malate is made from PEP, and its provision, in turn, requires the availability of carbohydrates. Because of the low capacity of the carbon-reduction cycle in guard cells, the needed reduced carbon must come from the mesophyll. It is transitorily stored as starch in the plastids. During stomatal opening, if the import rate of carbohydrates from the mesophyll does not suffice to deliver PEP at the required rate, products of starch breakdown will be exported from the plastids and made available for malate formation; guard-cell chloroplasts are green but appear to function like amyloplasts. (Lloyd recognized this particular situation as early as 1908 [Lloyd, 1908]). Obviously, guard-cell chloroplasts must possess mechanisms for an uptake of reduced carbon, for its deposition and its breakdown, and for the release of precursors of malate. During this investigation we were concerned solely with the mechanisms involved in import and export.

The major transport protein in the envelope of mesophyll chloroplasts is the phosphate translocator (Willey et al., 1991). It carries divalent phosphates in strict exchange and possesses particularly high affinities for Pi, PGA, and DHAP (Fliege et al., 1978). We wanted to find out whether guard-cell chloroplasts were equipped with a similar carrier and, if they were, whether the apparent function of guard-cell chloroplasts as amyloplasts had furthered the evolution of a phosphate translocator with a special substrate spectrum different from that of the corresponding mesophyll protein.

We developed a procedure for the isolation of guard-cell chloroplasts, differing from that of Preiss et al. (1985). It resulted in a suspension with a large fraction of viable plastids free from contamination by mesophyll chloroplasts. We were able to demonstrate that a phosphate translocator is active in guard-cell chloroplasts, and we determined its substrate specificity in uptake experiments in which organic phosphates competed with [^{32}P]Pi. (Overlach and Raschke, 1991).

MATERIALS AND METHODS

Plant Material

Plants of *Pisum sativum* L., Argenteum mutant (Jewer et al., 1982; Marx, 1982), were grown in a growth chamber with a light/dark cycle of 18 h/6 h and a temperature cycle of 20°C/17°C. Quantum flux was 200 μmol m^{-2} s^{-1}. The fully expanded pairs of leaflets from 4-week-old plants were harvested.

Preparation of Guard-Cell Chloroplasts

Isolation of intact guard-cell chloroplasts started with the preparation of guard-cell protoplasts according to the method

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Abbreviations: DHAP, dihydroxyacetone phosphate; GAPDH[NADP], NADP-dependent glyceraldehyde dehydrogenase; PEP, phospho*enol*pyruvate; PEPC, PEP carboxylase; PGA, 3-phosphoglycerate.

of Raschke and Hedrich (1989) with slight modifications. Figure 1 shows schematically how chloroplasts are prepared, especially how separation from mesophyll is achieved.

For each preparation 600 leaves were minced in a Waring blender in three bursts and incubated overnight in medium A (0.35 M mannitol, 5% [w/v] cellulase [Onozuka R-10], 0.15% [w/v] Macerozyme R-10, 10% [w/v] BSA, 10 mM sodium ascorbate, 1 mM calcium iminodiacetate, and 1.7 g L^{-1} penicillin-G [pH 5.5]). Guard-cell protoplasts set free were washed and filtered through three nylon nets (mesh width of 80, 20, and 14 μm) in succession, sedimented (10 min, 1400*g*), and resuspended in medium B (0.33 M sorbitol, 25 mM Hepes-K^+ [pH 7.2], 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, and 1.7 g L^{-1} penicillin-G).

Cell density was adjusted to 3.0×10^6 cells mL^{-1}. Contamination by mesophyll cells was examined by microscope and found to be less than 0.3% (cell/cell) for all preparations.

For the isolation of chloroplasts the protoplasts were ruptured by passing them twice through two nylon nets (6 and 5 μm) in sequence. The resulting chloroplast suspension was centrifuged for 3 min at 20*g* to remove starch granules. The plastids were sedimented by centrifugation (8 min, 2400*g*, 4°C) through a layer of 30% (v/v) Percoll in medium B. The sedimented chloroplasts were resuspended in medium B to give a final concentration of 3 μg of Chl mL^{-1}.

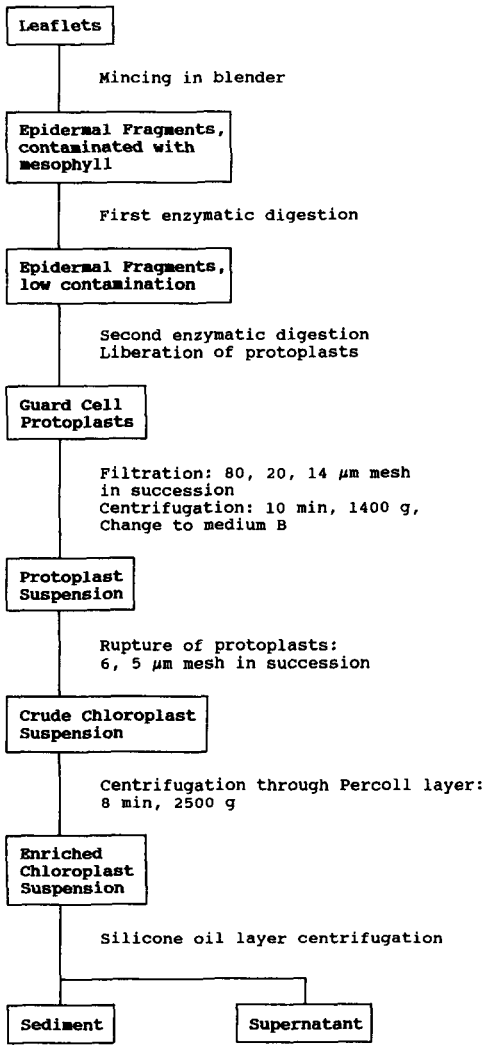


Figure 1. Flow diagram of the isolation procedure for guard-cell chloroplasts. For details see "Materials and Methods."

Measurements of Enzyme Activities

The purity of the chloroplast suspension was assessed by measurements of the activity of marker enzymes. The markers used were PEPC (EC 4.1.1.31) for the cytosol, its activity measured according to the method of Schnabl (1980); α-mannosidase (EC 3.2.1.24) for the vacuole, assayed according to the procedure of Ye The Li (1967); citrate synthase (EC 4.1.3.7) for mitochondria (Stitt, 1984); and GAPDH[NADP] (EC 5.3.1.1) for chloroplasts, as determined by the method of Wirtz et al. (1980). Except for the latency tests, all assay solutions contained 0.1% (w/v) Triton X-100.

Intactness of chloroplasts was controlled by determination of the latency of GAPDH[NADP] in the absence and presence of 0.1% (w/v) Triton X-100 (Journet and Douce, 1985).

Chl was measured according to the method of Arnon (1949).

Volume Determination and Phosphate Uptake

For the determination of chloroplast volume and for all transport experiments the method of silicone oil layer-filtering centrifugation was used (Heldt and Sauer, 1971). To determine the volume of a single plastid, the chloroplast medium was labeled with tritiated water (9.47 MBq μmol^{-1}) and [U-^{14}C]sorbitol (7.4 MBq μmol^{-1}). The radioactivity ratio of ^{3}H to ^{14}C was 3:1.

Phosphate transport was characterized using [^{32}P]Pi at concentrations between 0.05 and 1.0 mM; the respective specific activities were between 0.11 and 2.56 MBq μmol^{-1}. Transport was stopped after 10 s by centrifugation (30 s at full speed, Beckman Minifuge C; Beckman, Munich, FRG). Radioactivity was counted with a liquid scintillation counter. Time dependencies of phosphate uptake were determined at a concentration of 0.1 mM Pi in the medium. The inhibition of phosphate uptake by competing substrates was studied with concentrations of competing substrate between 0 and 1.0 mM (10 mM for Glc-1-P), at 0.05 and 0.1 mM external Pi. Transport was stopped after 10 s. All experiments were carried out at 15°C.

EM

Guard-cell protoplasts were fixed for 2 to 3 h in a solution consisting of 2% (w/v) glutaraldehyde, 12.5 mM potassium phosphate buffer (pH 7.0), 1 mM $CaCl_2$, 0.25% (v/v) tannic acid, and approximately 0.3 M mannitol. Postfixation was performed by adding 4% (w/v) OsO_4 in 50 mM potassium phosphate buffer (pH 7.0), after rinsing the prefixed protoplasts. The cells were stained with 5% (w/v) uranyl acetate and, after dehydration with acetone, with a saturated lead acetate solution in ethanol acetone.

Sections were mounted on grids, poststained with 0.3%

(w/v) lead citrate and 2% (w/v) uranyl acetate in methanol, and examined with 80 kV in a Phillips 400 electron microscope.

Chemicals

Cellulase and Macerozyme were purchased from Onozuka (Tokyo, Japan). ^3H_2O, [U-^{14}C]sorbitol, and [^{32}P]Pi were from Amersham Buchler (Braunschweig, FRG). Silicone oil was from Wacker Chemie (Munich, FRG).

RESULTS

Purity of the Chloroplast Suspension

Aliquots were taken during the preparation at the protoplast suspension step, before and after centrifugation through the Percoll layer and after silicone oil layer centrifugation to assess purity and intactness of the chloroplasts (Fig. 1). The enzyme activities measured in the protoplast suspension served as references and were set to 100% (Table I). Enzyme activities declined greatly during the preparation, but the loss was least in the activity of the chloroplast marker enzyme GAPDH[NADP]. The greatest divergence in activity loss appeared in the sediment after silicone oil layer-filtering centrifugation. Normalization of enzyme activities with respect to Chl content indicated that more than 60% of the original activity of the chloroplast marker enzyme appeared in the sediment, whereas only 1% of the initial PEPC activity appeared in the sediment, reflecting negligible contamination with cytosol. The remaining activities of α-mannosidase and particularly of citrate synthase were surprisingly high, disclosing the presence of vacuolar vesicles and mitochondria. Extension of centrifugation times did not improve the separation; to the contrary, increased amounts of nonchloroplastic marker activities appeared in the sediment.

Association of Mitochondria and Peroxisomes with Guard-Cell Chloroplasts

We interpreted the lack of success in removing all citrate synthase activity from the chloroplast suspension as evidence for a close association between chloroplasts and mitochondria in the guard cells. This notion received support from an inspection of guard-cell protoplasts by EM (Fig. 2). Mitochondria surround the chloroplasts in close proximity; in many instances they appear to produce impressions on the surface of the plastids. We presume, however, that the presence of mitochondria will not have caused grave errors in our investigation into the accumulation and the release of phosphates by the chloroplasts, the chloroplast volumes being much larger than those of the mitochondria (Fig. 2).

There was a paucity of peroxisomes (microbodies) in the guard cells. They were abundant in the mesophyll cells but appeared only occasionally in sections of guard cells; obviously their frequencies correlate with the dissimilar Rubisco activities in the two cell types. The lower electron micrograph of Figure 2 shows a microbody, placed between a plastid and a mitochondrion.

Intactness of the Plastids

Latency (Journet and Douce, 1985) of GAPDH[NADP] was determined by comparing activities measured in the absence and presence of 0.1% Triton X-100 (Table II). Centrifugation of the crude chloroplast suspension through the Percoll layer produced an increase in latency, indicating an enrichment of the fraction of intact chloroplasts by this step. Nevertheless, nearly half of all chloroplasts were broken. Because there was virtually no latency of GAPDH[NADP] left in the supernatant of the following silicone oil centrifugation (Table II), we assume that all of the intact chloroplast material had passed through the silicone oil layer, and the results of the

Table I. *Recovery of marker enzyme activity*

Relative enzyme activities are normalized with respect to the activities measured in the protoplast suspension. For each fraction, the first line shows the absolute recovery of the activity of a preparation from 600 leaflets, and the second line shows the data with respect to Chl content. For definition of fractions see Figure 1.

Fraction	GAPDH-[NADP] (n = 25)	α-Mannosidase (n = 5)	Citrate Synthase (n = 3)	PEPC (n = 8)
		Absolute activities (=100%)		
Protoplast suspension				
nmol min^{-1}	150	74	31	2010
nmol μg^{-1} min^{-1}	11	47	16	222
		Relative activities (%)		
Crude chloroplast suspension	80.3	55.4	64.8	79.7
	111.1	76.1	71.2	89.9
Enriched chloroplast suspension	11.4	17.3	17.1	1.8
	48.7	55.3	61.1	13.9
Sediment after silicone oil centrifugation	5.3	1.0	3.0	0.1
	63.7	19.0	34.8	1.2
Supernatant after silicone oil centrifugation	6.6	16.3	9.0	0.1

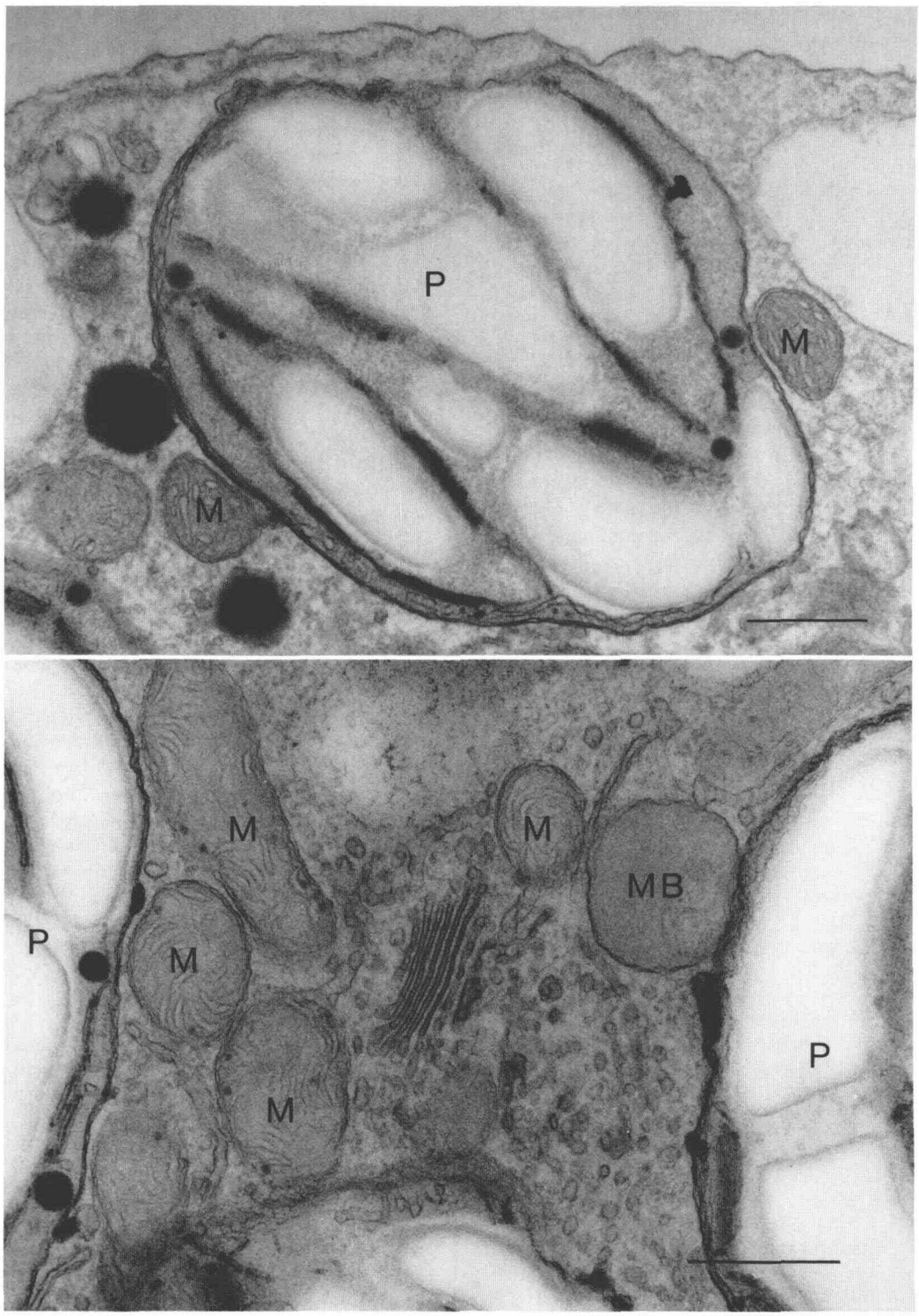


Figure 2. Electron micrographs of guard-cell protoplasts. Close association between chloroplasts (P) and mitochondria (M) in a guard-cell protoplast of *P. sativum*, as exhibited through EM. In the lower frame a microbody (MB) appears as well as a Golgi system. Bar = 0.5 μm.

Table II. *Latency data*

Intactness of chloroplasts, as indicated by the latency of GAPDH[NADP]. For definition of fractions see Figure 1. Data are the means of 25 determinations.

Fraction	Latency
	%
Protoplast suspension	81
Crude chloroplast suspension	27
Enriched chloroplast suspension	57
Sediment after silicone oil centrifugation	24
Supernatant after silicone oil centrifugation	3

uptake experiments were related to the fraction of intact plastids.

Volume of a Guard-Cell Chloroplast

The space inaccessible to [^{14}C]sorbitol but accessible to $^{3}H_2O$ represented the combined internal volume of the intact chloroplasts. Division of this volume by the number of complete chloroplasts per unit volume (as determined by microscope) yielded estimates of the volume of a guard-cell chloroplast; it was 40.3 fL (SD = 19.3 fL, based on 41 determinations). In electron micrographs of material fixed and embedded under isotonic conditions, guard-cell chloroplasts had a diameter of about 4.5 μm. If they had been completely spherical, the volume of a plastid would have been about 47.7 fL, supporting the estimate derived from our space determinations.

Phosphate Uptake and Its Kinetics

Phosphate uptake by guard-cell chloroplasts occurred rapidly (Fig. 3). Phosphate accumulation of [^{32}P]Pi saturated after about 5 min of transport. Provision of Pi at an external

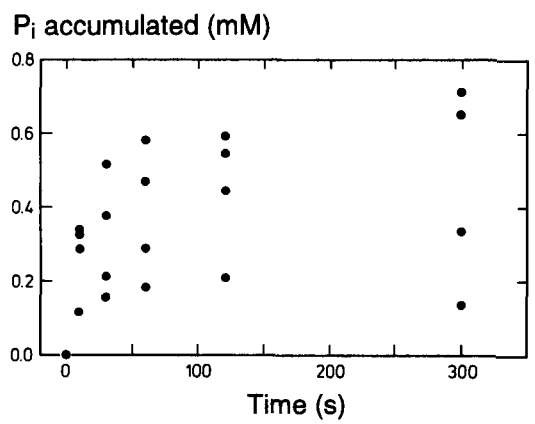
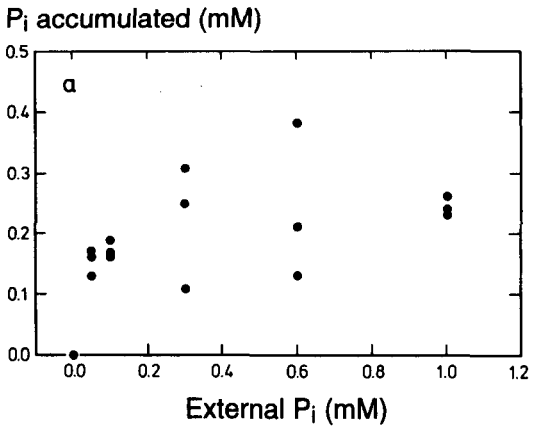


Figure 3. Time dependence of phosphate uptake. Shown is accumulated internal Pi concentration, as derived from measurements of radioactivity at different times. External Pi concentration was 0.1 mM. To demonstrate the scatter of data, data from four experiments are shown.



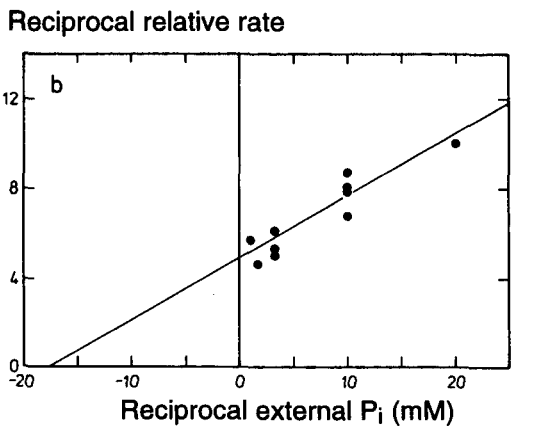


Figure 4. Kinetics of phosphate uptake. a, Dependence of phosphate accumulation on external Pi concentration was measured at six concentrations between 0.0 and 1.0 mM. Transport was stopped after 10 s. b, Lineweaver-Burk plot. Rates were normalized with respect to the uptake rate at 0.05 mM external Pi, which was set at unity. Data were taken from three experiments.

concentration of 0.1 mM resulted in an increase of the internal Pi concentration by 0.5 mM. The concentration increase of (labeled) Pi in the chloroplasts depended on the Pi concentration in the medium (Fig. 4a). From these data an affinity constant for Pi was determined according to Lineweaver-Burk plots and was found to be 0.05 mM, $n = 3$ (Fig. 4b). Transport rates for 0.1 mM external Pi were between 2 and 3 fmol h^{-1} per plastid during the first 10 s. We ascribe the magnitude of the scatter of data (as large as 50% of the mean) to some uncertainty in the latency estimates. The number of intact plastids may have been underestimated because it was determined at the end of each experiment.

Competition Experiments

The selectivity of the translocator was examined by the reduction of the rate of [^{32}P]Pi uptake in the presence of organic phosphates. As examples, the Lineweaver-Burk plots for the inhibition of Pi uptake for DHAP and Glc-6-P are shown in Figure 5. Inhibitor constants for various plastids for six compounds competing with the uptake of Pi are listed in

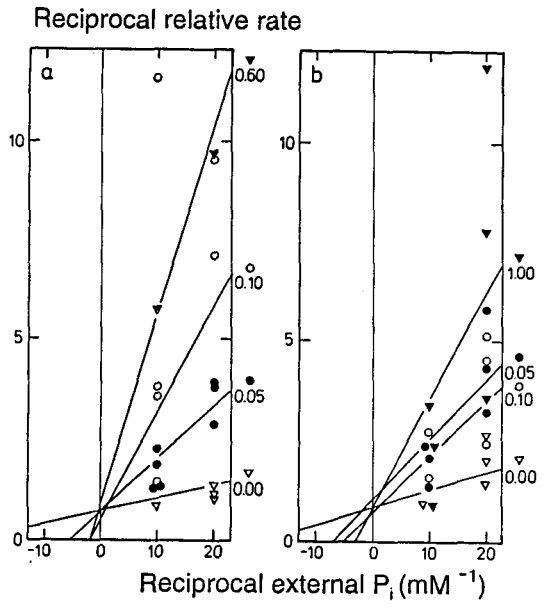


Figure 5. Inhibition of phosphate uptake by DHAP and Glc-6-P. Both panels show rates of uptake normalized with respect to the control rates without competing substrate, which were set at unity. Each concentration is represented by a different symbol. Linear regression was used to calculate the intercept with the abscissa. Four of six concentrations are shown. Lineweaver-Burk plots for DHAP (n = 4) (a) and Glc-6-P phosphate (n = 3) (b) are shown as competing substrates.

Table III. The first line of data shows the values we determined for guard-cell chloroplasts. The K_i values for DHAP and PGA were in the same order of magnitude as those determined for the phosphate translocator of other plastids, particularly for the mesophyll chloroplast of the C_3 plant *S. oleracea*. Obviously, a phosphate-uptake mechanism exists in guard-cell chloroplasts that functionally corresponds to the phosphate translocator of mesophyll chloroplasts. Surprisingly, Glc-6-P inhibited Pi uptake by the guard-cell plastids with a low K_i similar to that for the C_3 compounds. This property is not shared by other chloroplasts. For Glc-1-P the K_i value was one order of magnitude higher than that for Glc-6-P. The phosphate translocator of guard-cell chloroplasts has an affinity for Glc-6-P that is only a little lower than the affinities for DHAP and PGA. In this it resembles the phosphate translocator of the amyloplasts in roots (Borchert, 1990).

DISCUSSION

This investigation into the presence and properties of the phosphate translocator in guard-cell chloroplasts required the acceptance of a reasonable compromise among the diverging requirements of purity of the chloroplast suspension, intactness of the plastids, and yield of the preparation procedure. Damage and loss occurred not only during the rupture of the protoplasts but also during the separation steps. About one-half of the number of plastids was still intact in the final suspension. Nevertheless, it was possible, through the double-labeling procedure with ^3H_2O and [^{14}C]sorbitol, to determine the volume of the intact plastids and relate to it the accumulation of [^{32}P]Pi.

The activity of the marker enzymes α-mannosidase and citrate synthase (Table I) indicated that the chloroplast suspensions were contaminated with vacuolar vesicles and mitochondria. The vesicles incorporated the medium during chloroplast isolation. Therefore, these vesicles had the density of medium B and should not have been able to pass the silicone oil layer. Only 1% of the original vacuolar marker enzyme activity appeared in the sediment after silicone oil centrifugation. For this reason we did not correct for any vacuolar contribution to uptake or export. But rupture of vacuolar compartments has to be kept in mind when the phosphate concentration in the medium is estimated.

We also neglected any possible contribution of adhering mitochondria to uptake or export of phosphate because, in guard cells, their total volume was so much smaller than that of the chloroplasts (Fig. 2). In view of our inability to reduce the activity of citrate synthase in the enriched chloroplast fraction to values of less than 17% of the initial total activity (Table I), a finding of Preiss et al. (1985) attracts attention. These authors reported that, in isolated guard cells of *Commelina communis*, 18% of the activity of the mitochondrial marker enzyme Cyt *c* oxidase was located in the chloroplast fraction. Perhaps the association between chloroplasts and mitochondria is particularly close in guard cells. (We have begun looking into this possibility and are investigating its significance.)

We conclude that effects of broken plastids and contaminating vesicles and mitochondria can be disregarded in the results of our experiments. We can state that the fraction of complete chloroplasts in our preparations was able to accumulate labeled Pi to an internal concentration that reached a steady state of phosphate exchange when it was up to 5 times higher than the external concentration. Guard-cell chloroplasts of *P. sativum*, like other chloroplasts, possess a phosphate translocator. Its affinity for Pi, triose phosphates, and

Table III. *Constants for the competitive inhibition of Pi uptake through the phosphate translocator of various plastids*

The values for guard-cell chloroplasts are based on the data of this investigation; all others were taken from Heldt et al. (1990).

Plastid	Competing Substrate				
	DHAP	PGA	PEP	Glc-6-P	Glc-1-P
	mM				
P. sativum					
Guard-cell chloroplast	0.1	0.2	0.4	0.3	3.1
Root amyloplast	0.11	0.31	0.20	0.33	3.6
Spinacia oleracea					
Mesophyll chloroplast	0.13	0.15	4.7	40	n.d.[a]
Zea mays					
Mesophyll chloroplast	0.05	0.053	0.086	n.d.	n.d.
Panicum miliaceum					
Mesophyll chloroplast	0.4	0.72	0.48	n.d.	n.d.
Bundlesheath chloroplast	0.37	0.60	1.10	n.d.	n.d.

[a] n.d., Not determined.

PGA was similar to that of chloroplastic phosphate translocators from photosynthetic tissues of other species (Table III). However, the guard-cell phosphate translocator also differs from other translocators in that it possesses a measurable affinity for Glc-1-P and a large one for Glc-6-P. However, the affinity for Glc-1-P may have been only apparent in that it resulted from phosphoglucomutase activity. The affinity for Glc-6-P is as high as that determined for pea-root amyloplasts (Borchert et al., 1989).

The discovery of the ability of guard-cell chloroplasts to exchange Glc-6-P provides an answer to the question about the form in which they import reduced carbon. It was suggested that they take up PGA and triose phosphates (Preiss et al., 1985). If this is so, a difficulty arises: the path from C_3 compounds to starch requires the presence of a fructose-1,6-bisphosphatase in the guard-cell chloroplasts, but such an activity was determined to be virtually absent (guard-cell chloroplasts of *Vicia faba*, Hedrich et al., 1985). Now we recognize that, if carbohydrate were taken up from the cytosol as Glc-6-P, no fructose-1,6-bisphosphatase activity would be required in the guard-cell chloroplasts for starch formation. Import of Glc-6-P through the phosphate translocator would deliver carbon right to the intersection of the metabolic paths within the guard-cell chloroplasts that lead to starch synthesis, energy release through the oxidative pentose-phosphate cycle, and glycolysis.

Hite et al. (1992) demonstrated activities of the glycolytic ATP- and PPi-dependent phosphofructokinases in guard cells (of *V. faba*) that were 10 times higher, on a protein basis, than those in palisade cells. These high activities led Hite et al. to consider the operation of an additional translocator (for hexose phosphates) in the membrane of guard-cell chloroplasts.

Our current research is directed to the recognition of the form in which carbon is returned from the guard-cell chloroplasts to the cytosol through the phosphate translocator (and other carriers) and whether this form differs between conditions of darkness and light.

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LITERATURE CITED

Arnon DI (1949) Copper enzymes in isolated chloroplasts, polyphenoloxidase in *Beta vulgaris*. Plant Physiol **24:** 1–15

Borchert S (1990) Untersuchungen zum Stoffwechsel von nichtphotosynthetisch aktiven Plastiden aus Erbsenwurzeln. Doctoral Dissertation, Universität Göttingen, Göttingen, Germany

Borchert S, Große H, Heldt HW (1989) Specific transport of inorganic phosphate, glucose 6-phosphate, dihydroxyacetone phosphate and 3-phosphoglycerate into amyloplasts from pea roots. FEBS Lett. **253:** 183–186

Fliege R, Flügge UI, Werdan K, Heldt HW (1978) Specific transport of inorganic phosphate, PGA, and triosephosphates across the inner membrane of the envelope in spinach chloroplasts. Biochim Biophys Acta **502:** 232–247

Hedrich R, Raschke K, Stitt M (1985) A role of fructose-2,6-bisphosphate in regulating carbohydrate metabolism in guard cells. Plant Physiol **79:** 977–982

Heldt HW, Flügge UI, Borchert S (1991) Diversity of specificity and function of phosphate translocators in various plastids. Plant Physiol **95:** 341–343

Heldt HW, Flügge UI, Borchert S, Brückner G, Ohnishi JI (1990) Phosphate translocators in plastids. *In* I Zelitch, NS Allan, eds, Perspective in Biochemical and Genetic Regulation of Photosynthesis. AR Liss, New York, pp 39–54

Heldt HW, Sauer F (1971) The inner membrane of the chloroplast envelope as the site of specific transport. Biochim Biophys Acta **234:** 83–91

Hite DRC, Bodson MJ, Outlaw WH Jr (1992) Enzymic potential for fructose 6-phosphate phosphorylation by guard cells and by pallsade cells in leaves of the broad bean *Vicia faba* L. Histochem J **24:** 368–374

Jewer PC, Incoll LD, Shaw J (1982) Stomatal responses of Argenteum — a mutant of *Pisum sativum* L. with readily detachable leaf epidermis. Planta **155:** 146–153

Journet P, Douce R (1985) Enzymatic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. Plant Physiol **79:** 458–467

Li YT (1967) Studies on the glucosidase in jack bean meal. I: Isolation and properties of α-mannosidase. J Biol Chem **242:** 5474–5480

Lloyd FE (1908) The physiology of stomata. Carnegie Inst Washington Publ **82:** 1–142

Marx GA (1982) Argenteum (Arg) mutant of *Pisum*. Genetic control on breeding behavior. J Hered **73:** 413–420

Nelson SD, Mayo JM (1975) The occurrence of non-chlorophyllous guard cells in *Paphiopedilum* spp. Can J Bot **53:** 1–7

Outlaw WH Jr, Mayne BC, Zenger VE, Manchester J (1981) Presence of both photosystems in guard cells of *Vicia faba* L. Plant Physiol **67:** 12–16

Overlach S, Raschke K (1991) The phosphate translocator of isolated guard cell chloroplasts transports Glucose 6-Phosphate. (Abstr 316). Plant Physiol **96:** S-47

Preiss J, Robinson N, Spilatro S, McNamarra K (1985) Starch synthesis and its regulation. *In* RL Heath, J Preiss, eds, Regulation of Carbon Partitioning in Photosynthetic Tissue, Symposium in Plant Physiology. American Society of Plant Physiologists, Rockville, MD, pp 1–26

Raschke K, Hedrich R (1989) Patch clamp measurements on isolated guard cell protoplasts and vacuoles. Biomembranes, Part U: Cellular and subcellular transport: eucaryotic (Nonepithelial) Cells. Methods Enzymol **174:** 312–330

Reckmann U, Scheibe R, Raschke K (1990) Rubisco activity in guard cells compared with the solute requirement for stomatal opening. Plant Physiol **92:** 246–253

Schnabl H (1980) CO_2 and malate metabolism in starch-containing guard cell protoplasts. Planta **149:** 52–58

Stitt M (1984) Degradation of starch in chloroplasts: a buffer to sucrose metabolism. *In* DH Lewis, ed, Storage Carbohydrates in Vascular Plants. Cambridge University Press, Cambridge, UK, pp 205–230

Willey DL, Fischer K, Wachter E, Link TA, Flügge UI (1991) Molecular cloning and structural analysis of the phosphate translocator from pea chloroplasts and its comparison to the spinach phosphate translocator. Planta **183:** 451–461

Wirtz W, Stitt M, Heldt HW (1980) Enzymatic determination of metabolites in the subcellular compartments of spinach protoplasts. Plant Physiol **66:** 187–193

Zeiger E, Armond P, Melis A (1981) Fluorescence properties of guard cell chloroplast. Evidence for linear electron transport and light-harvesting pigments of photosystems I and II. Plant Physiol **67:** 17–20