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Molecular Characterization of a Carbon Transporter in Plastids from Heterotrophic Tissues: The Glucose 6-Phosphate/ Phosphate Antiporter

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Plastids of nongreen tissues import carbon as a source of biosynthetic pathways and energy. Within plastids, carbon can be used in the biosynthesis of starch or as a substrate for the oxidative pentose phosphate pathway, for example. We have used maize endosperm to purify a plastidic glucose 6-phosphate/phosphate translocator (GPT). The corresponding cDNA was isolated from maize endosperm as well as from tissues of pea roots and potato tubers. Analysis of the primary sequences of the cDNAs revealed that the GPT proteins have a high degree of identity with each other but share only ~38% identical amino acids with members of both the triose phosphate/phosphate translocator (TPT) and the phosphoenolpyruvate/phosphate translocator (PPT) families. Thus, the GPTs represent a third group of plastidic phosphate antiporters. All three classes of phosphate translocator genes show differential patterns of expression. Whereas the TPT gene is predominantly present in tissues that perform photosynthetic carbon metabolism and the PPT gene appears to be ubiquitously expressed, the expression of the GPT gene is mainly restricted to heterotrophic tissues. Expression of the coding region of the GPT in transformed yeast cells and subsequent transport experiments with the purified protein demonstrated that the GPT protein mediates a 1:1 exchange of glucose 6-phosphate mainly with inorganic phosphate and triose phosphates. Glucose 6-phosphate imported via the GPT can thus be used either for starch biosynthesis, during which process inorganic phosphate is released, or as a substrate for the oxidative pentose phosphate pathway, yielding triose phosphates.

INTRODUCTION

During C_3 photosynthesis, energy from solar radiation is used for the formation of phosphorylated C3 sugar phosphates, triose phosphates (trioseP), and 3-phosphoglycerate (3-PGA); these products are exported from the chloroplasts into the cytosol via the trioseP/3-PGA/phosphate translocator (TPT). In the mature leaves of most plants, the exported photosynthates are then used in the formation of sucrose, which is allocated via the phloem to the heterotrophic plant organs, such as young leaves, roots, seeds, fruits, or tubers. In these sink tissues, sucrose serves as a source of carbon and energy and is first cleaved by the action of invertases or sucrose synthase; the products of these reactions are converted into hexose phosphates.

Nongreen plastids of heterotrophic tissues are carbohydrate-importing organelles and, in the case of amyloplasts of storage tissues, the site of starch synthesis. Because these plastids are normally not able to generate hexose phosphates from C3 compounds due to the absence of fructose 1,6-bisphosphatase activity (Entwistle and ap Rees, 1988), they rely on the import of cytosolically generated hexose phosphates as the source of carbon for starch biosynthesis and, in addition, for the oxidative pentose phosphate pathway. The results of transport measurements with intact organelles or reconstituted tissues from different plants suggest that this transport is mediated by a phosphate translocator that imports hexose phosphates in exchange with inorganic phosphate or C3 sugar phosphates (Borchert et al., 1989, 1993; Hill and Smith, 1991, 1995; Neuhaus et al., 1993; Flügge and Weber, 1994; Schünemann and Borchert, 1994; Flügge, 1995; Schott et al., 1995; Quick and Neuhaus, 1996).

In nongreen tissues from most plants studied to date, glucose 6-phosphate (Glc6P) is the preferred hexose phosphate taken up by nongreen plastids. However, in amyloplasts from wheat endosperm, glucose 1-phosphate (Glc1P) rather than Glc6P is the precursor of starch biosynthesis (Tyson and ap Rees, 1988; Tetlow et al., 1994). Amyloplasts from potato tubers also appear to use Glc1P rather than Glc6P to support starch synthesis (Naeem et al., 1997), although previous

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experiments have shown that these plastids were able to transport Glc6P but not Glc1P (Schott et al., 1995).

The primary structure of the hexose phosphate/phosphate translocator from heterotrophic organs has to be quite different from that of the chloroplastic TPT because RNA gel blots showed that the latter is almost exclusively expressed in photosynthetic tissues but not in nongreen tissues (Fischer et al., 1997). Recently, we described a type of a plastidic phosphate translocator that is specific for the transport of phosphoenolpyruvate (PEP), the phosphoenolpyruvate/phosphate translocator (PPT; Fischer et al., 1997). The PPT is present both in heterotrophic and in photosynthetic tissues, but it does not transport hexose phosphates. Therefore, the nature of the phosphate translocator that transports hexose phosphates in exchange for inorganic phosphate remains elusive. In this study, we describe the isolation of a Glc6P/phosphate antiporter from maize endosperm and its molecular cloning and provide further characterization of corresponding DNAs from various plants.

RESULTS

Purification of the Glc6P/Phosphate Translocator from Maize Endosperm

Previous experiments have shown that plastidic envelope membranes from maize endosperm possess a high Glc6P/phosphate transport activity (Flügge, 1995). To purify the corresponding transporter of nongreen tissues, we used endosperm prepared from maize kernels at 14 days after pollination (DAP) as the initial material. A membrane fraction obtained from the homogenized material by differential centrifugation was subjected to a flotation centrifugation on a discontinuous sucrose gradient. The yellow membranes that rise to the 1.0 M/0.73 M sucrose interface were used for further separation and partial purification of the Glc6P/phosphate translocator (GPT) (Figure 1, lane 2).

After solubilization of the membranes, this preparation was loaded onto a HiTrap Q anion exchange column. The fraction of membrane proteins that did not bind to this column was subjected to heparin–Sepharose CL-6B chromatography. A protein fraction exhibiting a GPT transport activity in the reconstituted system was eluted at 90 mM NaCl (see Methods). As shown in Table 1, this procedure resulted in an ~200-fold increase of the specific GPT transport activity. The final fraction contained, besides a high molecular weight protein, two proteins with molecular masses of 31 and 30 kD (Figure 1, lane 3). Both proteins were excised from SDS–polyacrylamide gels and digested in situ with the endoproteinase Lys-C to gain information about the amino acid sequences of internal peptides.

A comparison of peptide sequences obtained with entries in protein sequence databases using the BLASTP program (Altschul et al., 1990) showed that the 30-kD protein represented a member of the porin family (Fischer et al., 1994a). Two peptide sequences were obtained from the 31-kD protein. One peptide (KVAVSFTHIIK, P2) was identical to sequences contained in the PPTs (Fischer et al., 1997), except for a substitution of one amino acid (Ile rather than Thr at position 9 in the peptide). The other peptide (KTQVVPVQSEGAQRLK, P1), however, lacked any significant homology to known proteins.

Molecular Cloning of GPTs from Nongreen Tissues and Protein Characteristics

On the basis of the P1 peptide sequence information, two degenerate oligonucleotides (gene-specific primers) were synthesized and used for the rapid amplification of cDNA ends (RACE; Schaefer, 1995). As a template for the first RACE reaction with one gene-specific primer and an adaptor oligo$(dT)_{15}$ oligonucleotide (RACE adaptor primer) primer, a first-strand cDNA from maize endosperm was used. It was synthesized by using poly$(A)^+$ RNA from maize endosperm as template and the race adapter primer (see Methods). The products of this first RACE reaction were subjected to a second polymerase chain reaction using nested primers (see Methods). We obtained a 1200-bp DNA fragment containing the oligonucleotide sequence corresponding to peptide P1. This fragment was then used to screen cDNA libraries from maize endosperm, pea roots, potato tubers, and cauliflower inflorescences.

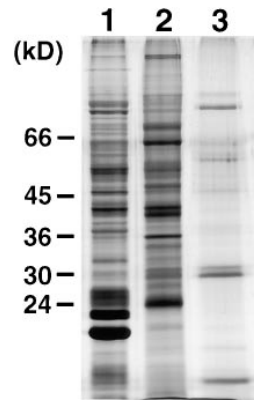

Figure 1. Preparation of the GPT from Maize Endosperm.

Maize endosperm was prepared from kernels and homogenized. The material was subjected to differential centrifugation followed by flotation centrifugation on a discontinuous sucrose gradient. The membrane fraction obtained at the 1.0 M/0.73 M sucrose interface was then used for purification of the GPT (see Methods, Results, and Table 1). Shown is the protein pattern obtained by SDS-PAGE of the initial material (lane 1), the membrane fraction obtained from the sucrose gradient (lane 2), and the fraction eluted from the heparin–Sepharose CL-6B column with 90 mM NaCl (lane 3). Molecular masses are indicated at left.

Table 1. Purification of the GPT from Maize Endosperm[a]

Preparation	Protein (mg)	Total Transport Activity (nmol min^{-1})	Specific Transport Activity (nmol mg^{-1} min^{-1})	Purification (-fold)
Tissue homogenate	1275	1350	1.1	1
Membrane fraction after sucrose density flotation centrifugation	10.4	27.4	2.7	2.5
Chromatography on Hi Trap Q-Heparin–Sepharose CL-6B	0.025	5.8	232	211

[a] Thirty-five grams of maize endosperm was used as the starting material, homogenized, and processed as described in Methods. Aliquots of each fraction were used for protein determination and for reconstitution into liposomes that had been preloaded with 25 mM Glc6P. ^{32}P-phosphate transport was measured as described in Methods.

From both the maize endosperm and the pea root cDNA libraries, several identical clones and one full-length cDNA were isolated. The cDNA from maize endosperm was 1647 bp and that from pea roots was 1609 bp, with coding regions of 1164 bp and 1203 bp, respectively. The coding regions correspond to 387 and 401 amino acid residues with predicted molecular masses of 42.4 kD (maize endosperm; ZmGPT13) and 43.7 kD (pea roots; PsGPT4). The sequence of the Lys-C peptide P1 was present in the N-terminal region of the mature ZmGPT13 protein, at amino acid residues 80 to 95 of the precursor protein, and the Lys-C peptide P2 was found at amino acid residues 181 to 191 of the precursor protein. A comparison of the cDNA sequences with entries in the EMBL nucleotide sequence database and the Swiss-Prot sequence database revealed no significant homologies with known proteins, except for a slight but significant similarity to the TPTs and the PPTs (see below).

We also obtained clones from potato tubers (missing some base pairs at the 5′ end) and cauliflower inflorescences, but they were not full length. Within the framework of the Arabidopsis genome sequencing project, sequences of chromosome 5 were recently released that contained the corresponding Arabidopsis GPT gene (accession number AB005232).

Analysis of the deduced GPT protein sequence revealed that these translocators are highly hydrophobic, with overall polarity indices of the deduced mature proteins of ~34% (Capaldi and Vanderkooi, 1972), as is the case for the TPTs and PPTs with overall polarity indices of 33 to 37% (Fischer et al., 1997). The hydrophobicity distribution analysis of the deduced amino acid sequences of the GPTs revealed approximately six membrane-spanning regions for the monomeric proteins, as is the case for the TPT and PPT proteins (Fischer et al., 1997). The predicted amino acid sequences of the GPT proteins from maize endosperm, pea roots, and Arabidopsis are presented in Figure 2 and compared with those of representatives of the TPTs (spinach, pea, and maize) and PPTs (maize, Arabidopsis, and tobacco).

The location of the processing sites in the TPT proteins had been determined previously by N-terminal amino acid sequencing of the isolated mature protein or by radiosequencing of the labeled and in vitro–processed radioactively labeled TPT precursor protein (Willey et al., 1991). The processing sites of the GPT precursor proteins were approximately deduced from sequence features of the TPTs that presumably are preserved among all plastidic phosphate translocators. The proteins possess transit peptides consisting of ~70 to 90 amino acid residues. The similarities between all translocators start with the first putative membrane spanning region that is located 20 to 30 amino acid residues downstream of the putative processing site. The similarities within this N-terminal region between all mature translocators are in fact rather low, but they are more pronounced within each group of translocators. In addition, the Lys-C peptide P1 derived from enzymatic digestion of the isolated GPT from maize endosperm is located in this region (amino acids 10 to 25) and should be contained in the mature part of the protein.

As is the case for the TPTs and PPTs, the GPT proteins share a high degree of identity with each other (mature proteins, 79 to 83%). However, the GPTs share only ~38% identical amino acids with members of both the TPT and the PPT families. Thus, the GPTs represent a third group of plastidic phosphate translocators. This conclusion was confirmed by constructing a phylogenetic tree by using the distance matrix method (Saitou and Nei, 1987). As shown in Figure 3, members of the three classes of transporters cluster at approximately equal distances from each other. The monocotyledonous plant maize branches off earlier, and the other dicotyledonous species cluster according to their taxonomic classification, for example, the TPTs from tobacco and potato (NtTPT and StTPT) as solanaceous species or the PPTs from Arabidopsis and cauliflower (AtPPT and BoPPT) as brassicaceous species, respectively. It was noted previously that despite the low overall similarities between the TPT and the PPT sequences, both classes of translocators contain five regions with remarkable similarity (50 to 95%; Fischer et al., 1997). Interestingly, these five regions of higher similarity can also be observed in the GPT proteins (Figure 2).

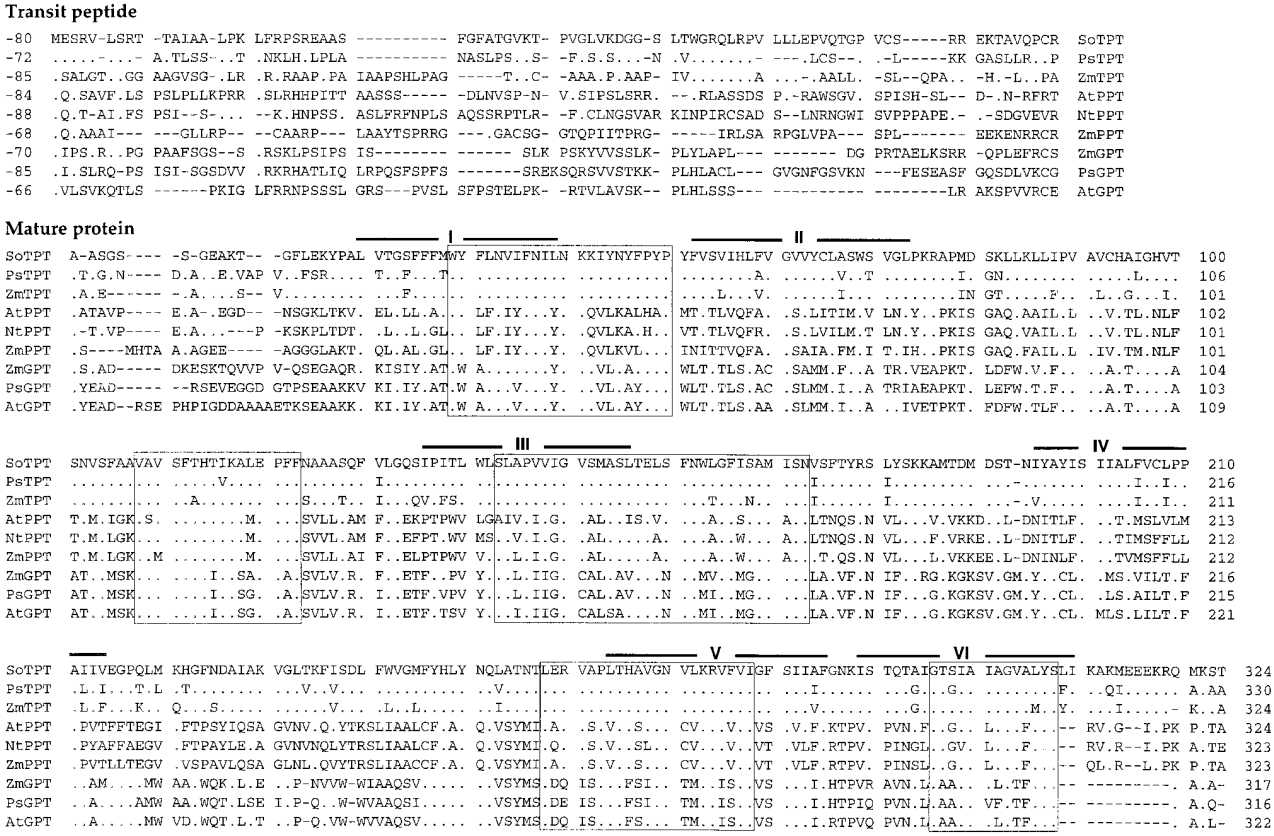

Figure 2. Alignment of Amino Acid Sequences of GPT Proteins with TPT and PPT Proteins.

GPT sequences from maize endosperm (ZmGPT), pea roots (PsGPT), and Arabidopsis (AtGTP, genome sequencing project) are aligned with TPT sequences from spinach (SoTPT; Flügge et al., 1989), pea (PsTPT; Willey et al., 1991), and maize (ZmTPT; Fischer et al., 1994b) and PPT sequences (Fischer et al., 1997) from Arabidopsis (AtPPT), maize (ZmPPT), and tobacco (NtPPT). Identities of amino acids between the translocators are indicated by dots. Amino acids are numbered beginning with the first amino acid of the mature proteins. Dashes indicate gaps introduced to maximize alignment. The locations of the putative six membrane-spanning α-helices are indicated (I to VI). Five regions of high homology between all translocators are boxed. The GenBank accession numbers for ZmGPT and PsGPT cDNA sequences are AF020813 and AF020814, respectively, and the one for the GPT cDNA sequence from potato tubers (data not shown) is AF020816.

Transport Characteristics of the GPT Protein

The function of a translocator can be characterized best by expressing the corresponding cDNA in a heterologous system, for example, yeast cells. Because, in the case of yeast cells, plant plastidic membrane proteins are directed into internal cell membranes, these membranes, or respectively, the protein isolated therefrom, have to be reconstituted into artificial membranes for subsequent determinations of the transport characteristics (Loddenkötter et al., 1993; Fischer et al., 1997). If the translocator protein contains an affinity tag, the protein can be purified to homogeneity from the membranes by affinity chromatography.

To study the transport characteristics of the GPT protein, the GPT cDNA encoding the mature part of the GPT was expressed in yeast cells. As outlined above, the exact position of the processing site was not definitely determined. This, however, appears to be of minor importance, because previous experiments had shown that the production of a functional plastidic transport protein with transport characteristics identical to those of the authentic protein can also be achieved by using the whole precursor protein (Weber et al., 1995). The experiments were performed with the GPT cDNA from pea roots rather than that from maize endosperm, because cDNAs from monocotyledonous species could not be functionally expressed in yeast cells (B. Kammerer and U.-I. Flügge, unpublished observations).

The GPT cDNA from pea roots was fused to a DNA fragment encoding a His_6-tag and subsequently subcloned into the yeast expression vector pEVP11. This construct was used to transform cells from the fission yeast *Schizosaccharomyces pombe*, and GPT was subsequently isolated to

homogeneity from the membrane fraction by Ni^{2+}–nitrilotriacetic acid chromatography and reconstituted into liposomes. Table 2 shows the substrate specificities of the GPT reconstituted into liposomes preloaded with different phosphorylated metabolites that function as exchangeable countersubstrates. For comparison, the substrate specificities of the histidine-tagged TPT and PPT proteins are also listed in Table 2.

Evidently, inorganic phosphate, trioseP, and Glc6P are about equally well accepted as countersubstrates by the GPT. To a lesser extent, transport of ^{32}P-phosphate was also supported by liposomes that had been preloaded with 3-PGA, whereas PEP only serves as a poor countersubstrate. Both Glc1P and fructose 6-phosphate (Frc6P) obviously cannot be transported by the GPT. These data are corroborated by measurements of the apparent kinetic constants of the GPT for the transport of ^{32}P-phosphate and ^{14}C-Glc6P. These data are listed in Table 3. The K_m(app) (phosphate) and the K_m(app)(Glc6P) values were determined to be 1.1 and 0.7 mM, respectively, and are comparable to the corresponding apparent inhibition constants (K_i values of 0.6 to 1.1 mM) and the K_m(app)(phosphate) values of both the TPT and the PPT (0.8 to 1.0 mM; Fischer et al., 1997). The apparent K_i values of Glc6P and phosphate for the transport of ^{32}P-phosphate and ^{14}C-Glc6P, respectively, are 0.6 and 1.1 mM and thus are in the range of the corresponding apparent K_m values.

Table 2. Determination of Substrate Specificities of Recombinant Phosphate Translocators from Heterotrophic Tissues and Chloroplasts Expressed in Yeast Cells

	Recombinant (Histidine-Tagged) Translocator Proteins Purified from *S. pombe* Cells[b]		
Internal Liposomal Substrate[a]	GPT Pea Root Plastids	PPT Cauliflower Bud Plastids[c]	TPT Spinach Chloroplasts[c]
Phosphate	=100	=100	=100
TrioseP	112 ± 5	22 ± 3	92 ± 17
3-PGA	50 ± 1	16 ± 3	90 ± 16
PEP	20 ± 1	72 ± 9	5 ± 2
Glc6P	90 ± 9	2 ± 1	5 ± 4
Glc1P	<1	4 ± 5	ND[d]
Frc6P	<1	ND	ND

[a] The liposomes had been preloaded with 25 mM substrates as indicated.

[b] The transport activities of the pea root GPT–His_6, the cauliflower PPT–His_6, and the spinach TPT–His_6 proteins, respectively, that had been purified to apparent homogeneity by Ni^{2+}–nitrilotriacetic acid chromatography from *S. pombe* cells were reconstituted into the liposomes. Transport of ^{32}P-phosphate was measured as described in Methods and is given as a percentage of the activity measured for proteoliposomes preloaded with inorganic phosphate (minus values obtained for liposomes containing only buffer). The 100% exchange activities (micromoles per milligram of protein per minute) of the recombinant proteins were 1.2 (GPT), 1.5 (PPT), and 0.85 (TPT), respectively. Mean values are from three to five different experiments ±SE.

[c] Data from Fischer et al. (1997).

[d] ND, not determined.

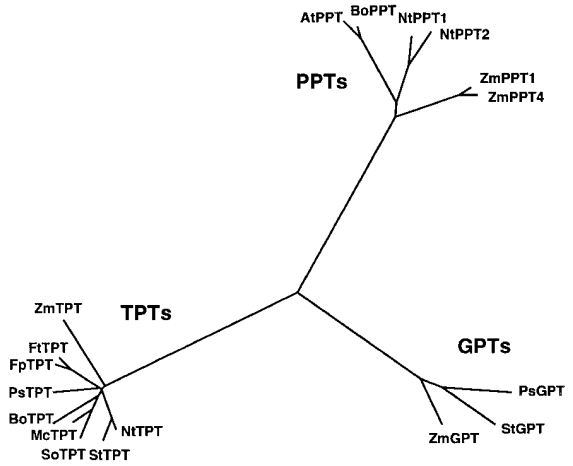

Figure 3. Phylogenetic Tree of the Phosphate Translocator Family Using the Maximum Likelihood of the Phylogenetic Analysis Program Package.

The tree was constructed on the basis of the available TPT, PPT, and GPT amino acid sequences. The alignment of the sequences was created using the CLUSTAL W program and used for constructing a phylogenetic tree (Higgins et al., 1992). From this treefile, an unrooted tree was drawn using the Drawtree program from the PHYLIP 3.572 program package. At, Arabidopsis; Bo, cauliflower; Fp, *Flaveria pringlei*; Ft, *Flaveria trinervia*; Mc, *Mesembryanthemum crystallinum*; Nt, tobacco; Ps, pea; So, spinach; St, potato; Zm, maize.

The low K_i values of trioseP for the transport of both ^{32}P-phosphate and ^{14}C-Glc6P indicate that trioseP can effectively compete with these substrates for binding to the GPT. The K_i (3-PGA) values for the transport of phosphate and Glc6P are approximately twice as high, and the K_i (PEP) values are three to 10 times higher than the corresponding K_m values.

These data again indicate that 3-PGA is a poorer substrate of the GPT compared with phosphate and Glc6P and that PEP, under physiological conditions in which it has to compete with the other phosphorylated metabolites for binding to the GPT, is almost never transported by the GPT. Thus, the GPT represents a plastidic phosphate translocator with the ability to transport preferentially Glc6P and trioseP but also 3-PGA. This is in contrast to the transport characteristics of the TPT and the PPT (Table 2). Besides inorganic phosphate, the TPT transports only trioseP and 3-PGA, whereas the PPT mediates the exchange of inorganic phosphate for PEP.

To determine the stoichiometry of the Glc6P/phosphate exchange mediated by the affinity-purified GPT, we measured

Table 3. Apparent K_m (Phosphate), K_m (Glc6P), and K_i Values of the Recombinant GPT from Pea Roots for Various Phosphorylated Metabolites

	Kinetic Constants for the Transport of	
Substrate	^{32}P-Phosphate[a]	^{14}C-Glc6P[a]
	K_m (app) (mM)	
	1.1 ± 0.1	0.7 ± 0.1
	K_i (app) (mM)	
Phosphate	none	0.6 ± 0.1
Glc6P	1.1 ± 0.1	none
TrioseP	0.6 ± 0.1	0.4 ± 0.1
3-PGA	1.8 ± 0.1	1.3 ± 0.1
PEP	2.9 ± 0.2	<7

[a] The transport activites of ^{32}P-phosphate and ^{14}C-Glc6P, respectively, were measured essentially as described in Methods and in footnote b of Table 2. All experiments were performed with liposomes that had been preloaded with 25 mM inorganic phosphate. Mean values are from three different experiments ±SE.

the influx of ^{14}C-Glc6P into phosphate-containing liposomes and compared it with the efflux of ^{32}P-phosphate from ^{32}P-phosphate–preloaded liposomes (see Methods). Loading of the liposomes with ^{32}P-phosphate was performed after the GPT had been incorporated into the liposomal membranes. This procedure ensures that ^{32}P-phosphate is taken up only by protein-containing liposomes from which it can be released after the subsequent addition of unlabeled Glc6P. Figure 4 shows the time-dependent export of ^{32}P-phosphate after the addition of externally added Glc6P (1.3 mM) and the time course of the ^{14}C-Glc6P import into phosphate-preloaded liposomes. In five independent experiments, the stoichiometry for phosphate/Glc6P exchange was close to 1 (0.99 ± 0.09). Similar results were obtained when ^{14}C-Glc6P–preloaded liposomes were used, and the release of ^{14}C-Glc6P was correlated with the uptake of ^{32}P-phosphate into these liposomes (data not shown). Thus, the antiport mediated by the GPT proceeds via a 1:1 exchange mechanism.

Members of the Phosphate Translocator Family Display Differential Patterns of Expression

To determine the expression patterns of the GPT in comparison with those of the TPT and the PPT, RNA gel blot analysis was performed with RNA samples from maize leaf, stems, developing stamens (filament and anthers), tassels, silks, and developing kernels. Figure 5A shows that the phosphate translocator gene classes have distinctly different profiles of organ expression. As shown earlier (Schulz et al., 1993; Fischer et al., 1997), the TPT is expressed predominantly in photosynthetic tissues and is absent in nongreen tissues except for the male reproductive organs (tassels and stamens). The PPT mRNA could be detected in all tissues tested. Transcripts of the GPT gene were almost lacking in leaves; however, high levels of GPT steady state mRNA were present in roots and the reproductive organs. In addition, high levels of GTP-specific transcripts were also present in developing kernels up to 20 DAP, with low levels present only before pollination (Figure 5B). The expression of the GPT gene was also studied in potato (Figure 5C). In the tissues examined (from leaves, stems, sepals, flowers, and tubers), only tubers had high levels of GPT mRNA. In pea, the GPT gene was highly expressed in pods and roots, but almost no GTP-specific transcripts were detected in photosynthetic tissues (data not shown).

Thus, the three classes of phosphate translocator genes show differential patterns of expression. Whereas the TPT gene is predominantly present in tissues that perform photosynthetic carbon metabolism and the PPT gene appears to be ubiquitously expressed, the expression of the GPT gene is restricted mainly to heterotrophic tissues.

Import of the GPT into Nongreen Plastids and Chloroplasts

Because the GPT is present mainly in heterotrophic tissues, we studied the in vitro import of the GPT precursor protein

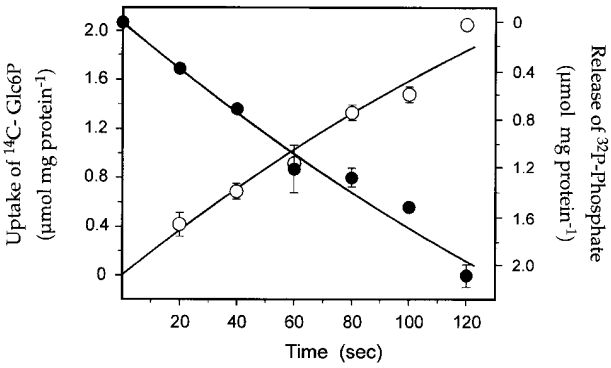

Figure 4. Uptake of Glc6P via the GPT Proceeds by a 1:1 Counterexchange with Inorganic Phosphate.

The recombinant GPT was isolated from yeast cells and reconstituted into liposomes that had been preloaded with inorganic phosphate (final concentration of 20 mM). After freezing and thawing, half of the liposomes were incubated for 40 min at 20°C with ^{32}P-phosphate (final specific activity of 9000 cpm/nmol). The external radioactivity was subsequently removed by chromatography on a Sephadex G-25 column. Unlabeled Glc6P (final concentration of 1.3 mM) was then added, and the release of ^{32}P-phosphate was determined at the indicated time intervals. The other half of the liposomes were used in ^{14}C-Glc6P uptake studies (final specific activity of 6000 dpm/nmol). For details, see Methods. Closed circles, release of ^{32}P-phosphate initiated by the addition of unlabeled Glc6P; open circles, time course of the uptake of ^{14}C-Glc6P. Means are of five different experiments.

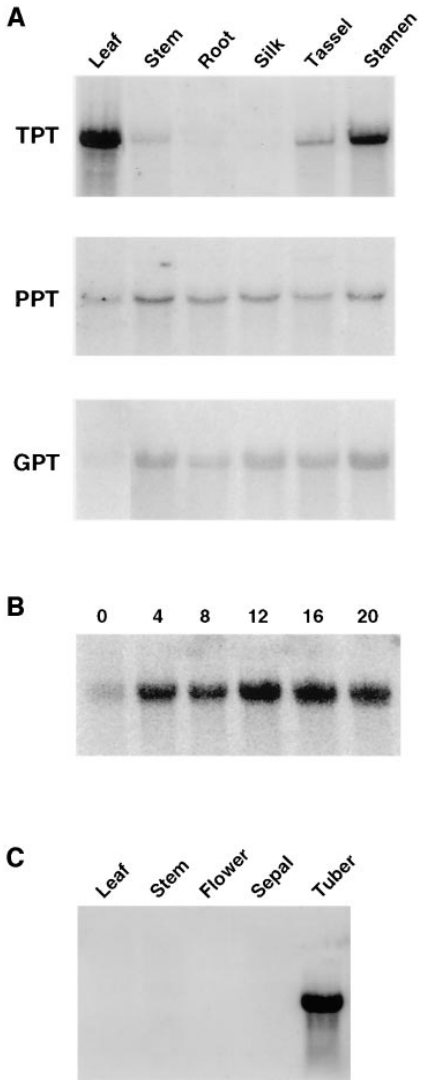

Figure 5. Expression of GPT Gene in Plant Tissues.

(A) RNA gel blot analysis of TPT, PPT, and GPT, and GPT RNA of maize. Fifteen micrograms of total RNA isolated from source leaves, stems, roots, elongating silks, tassels, and stamens (developing anthers and filaments) was hybridized with the respective TPT, PPT, and GPT cDNA probes from maize (TPT-mb2 [Fischer et al., 1994b] and MzrPPT1 [Fischer et al., 1997], respectively) after gel electrophoresis and subsequent transfer of the RNA to nylon membranes.
(B) RNA gel blot analysis of GPT mRNA of developing maize kernels. The kernels were harvested at the indicated days after pollination. Total RNA was isolated from the kernels and analyzed (15 μg per lane) as given in **(A)**.
(C) RNA gel blot analysis of GPT mRNA of potato. Total RNA was isolated from leaves, stems, whole flowers, sepals, and young tubers and analyzed (15 μg per lane) as given in **(A)**.
The hybridization conditions are described in Methods.

into plastids of this type of tissue. In vitro–synthesized GPT protein from pea roots was added to nongreen plastids. For this type of experiment, we used plastids from cauliflower buds rather than plastids from pea roots because, on the one hand, the import machinery appears to be conserved among different types of organelles (Klösgen et al., 1989); on the other hand, previous studies have shown that cauliflower bud plastids can be used successfully for protein import experiments into nongreen plastids (Fischer et al., 1994a, 1997). The import of the GPT protein was performed at 0 and 25°C, respectively, and the results of the corresponding experiments are shown in Figure 6A. Only binding of the precursor protein (apparent molecular mass of 43 kD) was observed in the absence of external ATP (Figure 6A, lanes 2 and 5). Externally added ATP resulted in import and a two-step processing of the precursor protein, yielding an

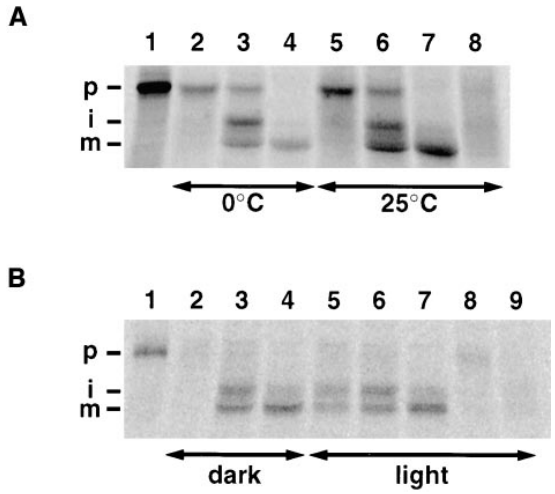

Figure 6. Import of the ^{35}S-Labeled GPT Precursor Protein into Nongreen Plastids and Chloroplasts.

The plastids (5.6 mg of protein mL^{-1} [cauliflower bud plastids] or 0.66 mg of chlorophyll mL^{-1} [pea chloroplasts]) were preincubated for 15 min in import buffer (see Methods).
(A) Import into cauliflower bud plastids.
(B) Import into pea chloroplasts.
Import was performed at 0°C (**[A]**, lanes 2 to 4), at 25°C (**[A]**, lanes 5 to 8), in the dark (**[B]**, lanes 2 to 4), or in the light (**[B]**, lanes 5 to 9) and in the presence of 10 units of apyrase (**[A]** and **[B]**, lanes 2), 5 μM valinomycin, 5 μM carbonylcyanide *m*-chlorophenylhydrazone (**[B]**, lanes 2 and 8), and 2 mM ATP (**[A]**, lanes 3, 4, and 6 to 8; **[B]**, lanes 3, 4, 6, 7, and 9). Sample 8 (**[A]**, lane 8) and sample 9 (**[B]**, lane 9) contained plastids that had been pretreated with thermolysin (30 μg mL^{-1}). After import, samples 4 and 7 (**[A]** and **[B]**, lanes 4 and 7) were further treated with thermolysin (50 μg mL^{-1}) in the presence of 1 mM $CaCl_2$ for 30 min. Envelope membranes were isolated as described earlier (Flügge et al., 1989) and analyzed by SDS-PAGE and fluorography. Lanes 1 (**[A]** and **[B]**), in vitro–synthesized precursor proteins. p, i, and m represent the precursor protein, the intermediate form, and the mature protein, respectively.

intermediate and the mature form with apparent molecular masses of 35 and 32 kD, respectively (Figure 6A, lanes 3 and 6). As expected, the import efficiency was appreciably higher at 25°C compared with 0°C. As shown by the subsequent thermolysin treatment of the plastids, the processed mature GPT protein was inserted into the plastidic envelope membrane in a protease-resistant manner, whereas the intermediate was accessible to the externally added protease (Figure 6A, lanes 4 and 7). If the plastids were pretreated with protease, binding and import were strongly suppressed, indicating that proteinaceous receptors are required for the binding process (Figure 6A, lane 8).

We also studied the import of the pea GPT precursor protein into isolated pea chloroplasts (Figure 6B), which subsequently were fractionated into a soluble fraction and fractions containing thylakoids and envelope membranes, respectively (Flügge et al., 1989). As shown for the TPT and PPT proteins (Flügge et al., 1989; Fischer et al., 1997), the imported GPT protein could be found only in the envelope compartment. Insertion into the envelope membrane under both dark and light conditions was strictly dependent on ATP; no import was observed if either ATP was omitted (Figure 6B, dark conditions, lane 2) or the production of photosynthetically generated ATP was prevented by uncouplers (Figure 6B, lane 8). Subsequent treatment of the chloroplasts with the protease thermolysin revealed that the processed mature form was protease resistantly inserted into the envelope membrane (Figure 6B, lanes 4 and 7). Pretreatment of the chloroplasts with thermolysin led to a complete loss of binding and import, again indicating the requirement for proteinaceous surface receptors (Figure 6B, lane 9). As is the case for the import of the pea GPT precursor protein into nongreen plastids, the processing of the pea GPT precursor protein into chloroplasts occurs via an intermediate (apparent molecular mass of 35 kD). Most likely, the intermediate arose from cleavage within the GPT transit peptide. Such a two-step processing of a plastidic membrane protein has also been observed for the Bt1 protein, a putative adenylate translocator of the inner envelope membrane from maize amyloplasts (Li et al., 1992). Overall, the GPT can be imported not only into nongreen plastids but also into photosynthetically active plastids.

DISCUSSION

In this study, we describe the purification of the GPT protein from maize endosperm and the subsequent isolation of corresponding cDNAs from different plants. We used maize endosperm as starting material for the preparation of the protein because this tissue has been shown to possess a highly active GPT (Flügge, 1995). The GPT protein was purified from a maize endosperm membrane fraction that was obtained by sucrose density flotation centrifugation and that contained an enriched Glc6P/phosphate exchange activity (Table 1). The sequence information from peptides obtained from a 31-kD protein enabled us to isolate corresponding cDNA clones from maize endosperm, pea roots, potato tubers, and cauliflower inflorescences.

The alignment of the mature GPT proteins from various plants revealed an ~38% identity to the two already known classes of plastidic phosphate antiporters, the TPTs and the PPTs. Also, the phylogenetic tree, constructed on the basis of the available TPT, PPT, and GPT amino acid sequences, showed that the GPTs can be classified as a new group of plastidic phosphate antiporters (Figure 3). The mature parts of all three classes of phosphate translocators consist of ~317 to 330 amino acid residues and contain, remarkably, five regions of high similarity (Figure 2). This observation suggests that these conserved regions are of particular relevance for the transport function of these translocators. One of these regions contains a cluster of two positively charged amino acid residues (Lys-273 and Arg-274 of the spinach TPT). This cluster is presumably involved in substrate binding (Fischer et al., 1994b).

To study the transport function of the GPT in more detail, the GPT from pea roots was chosen as a representative and produced as a histidine-tagged protein in yeast cells. The recombinant protein was purified to homogeneity from these cells by affinity chromatography and reconstituted into artificial membranes. These experiments clearly revealed that the transport function of the GPT is different from those of both the TPT (mediating the transport of inorganic phosphate, trioseP, and 3-PGA) and the PPT as a PEP/phosphate antiporter (Tables 2 and 3). The GPT accepts both Glc6P and trioseP as exchangeable countersubstrates for inorganic phosphate. It has a lower affinity toward 3-PGA, which is in accordance with a higher K_i (3-PGA) value compared with the corresponding K_m values for the transport of phosphate and Glc6P. However, if one assumes that the concentration of 3-PGA in the tissue is at least one order of magnitude higher than that of trioseP (Liu and Shannon, 1981), 3-PGA can be regarded as an additional metabolite that is transported by the GPT under physiological conditions. The transport of PEP by the GPT might actually be negligible, and other hexose phosphates, such as Glc1P and Frc6P, are virtually not transported by the GPT.

A translocator that is specific for the transport of phosphate, trioseP, 3-PGA, PEP, and Glc6P but not of Glc1P has been described for a number of nongreen tissues, that is, pea roots (Borchert et al., 1989, 1993), pea embryos (Hill and Smith, 1995), cauliflower inflorescences (Flügge and Weber, 1994), maize endosperm (Flügge, 1995), potato tubers (Schott et al., 1995), and pepper fruits (Quick and Neuhaus, 1996). A general hexose phosphate translocator that transports Glc6P and, in addition, Glc1P has been demonstrated to exist in tomato fruits (Schünemann and Borchert, 1994) and wheat endosperm (Tetlow et al., 1996), or the presence of such an activity has been deduced indirectly from the observation of Glc1P-dependent starch synthesis, for example, in wheat endosperm (Tyson and ap Rees, 1988), maize

endosperm (Neuhaus et al., 1993), and potato tubers (Naeem et al., 1997).

Until recently, many believed that these transport processes are mediated by a TPT-like phosphate translocator that also accepts the other phosphorylated metabolites as substrates. Our findings clearly show that these metabolites are not transported by a single transport system that is able to mediate the transport of the metabolites mentioned before but rather by a set of different members of the phosphate translocator family with partially overlapping substrate specificities. The TPT is obviously absent in nongreen tissues. In these tissues, the transport of trioseP (and of 3-PGA) can proceed by an exchange with either Glc6P or inorganic phosphate, whose processes are mediated by the GPT. Under physiological conditions, PEP is not transported by the GPT but rather is transported by the recently discovered PPT that is present in all tissues, although its transcripts are more abundant in nongreen tissues (Fischer et al., 1997). The identity of the hexose phosphate transporter that is specific for the uptake of both Glc6P and Glc1P remains to be determined.

As shown in Table 2, Glc6P is transported mainly in exchange for inorganic phosphate and trioseP. In nongreen plastids of starch-storing tissues, the imported Glc6P can be used as the substrate for the oxidative pentose phosphate pathway, yielding trioseP as a countersubstrate for the GPT. The reducing equivalents formed by this metabolic pathway are required for the reduction of nitrite and for the biosynthesis of amino acids (Bowsher et al., 1992). On the other hand, the imported Glc6P can serve as the precursor for starch biosynthesis. It is subsequently converted to Glc1P, which is the substrate for the ADP–glucose pyrophosphorylase, yielding pyrophosphate (PPi) and ADP–glucose as the substrate for starch synthases. PPi is cleaved by a stromal pyrophosphatase, yielding two molecules of inorganic phosphate. Thus, two molecules of phosphate have to be released from the plastids in exchange for every one molecule of Glc6P that is taken up. Analysis of the stoichiometry of the Glc6P/phosphate exchange as mediated by the GPT revealed a 1:1 ratio (Figure 4). To prevent an accumulation of stromal inorganic phosphate, the second molecule of inorganic phosphate has to be removed from the plastids by an as yet unknown mechanism.

We showed earlier that the TPT can switch from an antiport to a uniport mechanism if high substrate concentrations on both sides of the membrane are provided (Schwarz et al., 1994). It remains to be elucidated whether the GPT can also behave as a potential uniporter. On the other hand, it has been assumed that inorganic phosphate can be transported unidirectionally; however, its maximum transport rates are two orders of magnitude lower compared with that of the antiport mode (Neuhaus and Maas, 1996).

Taken together, these data show that the GPT links the cytosolically located conversion of sucrose and hexoses to Glc6P with metabolic reactions within the plastid, that is, the biosynthesis of starch and the oxidative pentose phosphate pathway that delivers reduction equivalents for the reduction of nitrite and the biosynthesis of amino acids. Its function in nongreen tissues is depicted in Figure 7.

As expected from the physiological function of the GPT protein, GPT-specific transcripts are barely detectable in photosynthetic tissues but are abundant in heterotrophic tissues that utilize Glc6P for starch synthesis, for example, potato tubers, maize kernels, and pea roots. For the developing endosperm from maize and barley, it has been shown recently that the key enzyme for starch biosynthesis, ADP–glucose pyrophosphorylase, is present mainly in the cytosol rather than in the plastids (Denyer et al., 1996; Thorbjørnsen et al., 1996). The cytosolically formed ADP–glucose is presumably the substrate for an ADP–glucose/adenylate antiporter and, after being imported into the plastids, for the starch synthases. Thus, the need for the import of Glc6P (and of ATP) for starch biosynthesis can be bypassed in these tissues by an import of ADP–glucose into the amyloplasts. Most probably, the Bt1 protein, the cDNA of which has already been cloned, serves the function of the ADP–glucose/adenylate translocator (Sullivan et al., 1991; Sullivan and Kaneko, 1995). Whether the GPT and the ADP–glucose/adenylate translocator are alternatively expressed in different cell types of the maize kernel and/or whether both genes are differentially expressed during endosperm development remains to be determined.

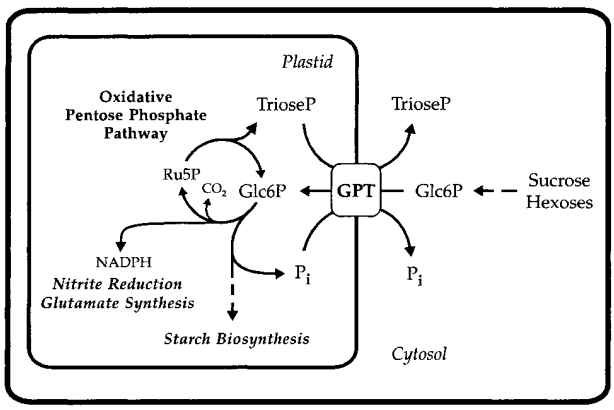

Figure 7. Proposed Function of the GPT Protein in Heterotrophic Tissues.

Sucrose and hexoses, delivered from source tissues, are converted to Glc6P that is imported into the nongreen plastids via the GPT. Within the plastids, Glc6P can be transformed to trioseP via the oxidative pentose phosphate pathway in which redox equivalents are delivered for the reduction of nitrite and glutamate synthesis. In addition, Glc6P is the precursor for starch biosynthesis, during which process inorganic phosphate is released. Both substrates, trioseP and inorganic phosphate, can be used as countersubstrates for the GPT. Glc6P, glucose 6-phosphate; P_i, inorganic phosphate; Ru5P, ribulose 5-phosphate; TrioseP, triose phosphates.

The data presented here demonstrate that the GPT protein can also be imported into photosynthetically active plastids (Figure 6); likewise, low expression of the GPT gene can be detected in green tissues (Figure 5). The reason for the observed low abundance in green tissues might be that the GPT activity is restricted to specialized cells. It has been shown that the envelope of guard cell chloroplasts contains a GPT-like transport activity (Overlach et al., 1993). Because of the absence of fructose 1,6-bisphosphatase activity (Hedrich et al., 1985), these chloroplasts rely on the supply of hexose phosphate for the biosynthesis of starch, as is the case for nongreen plastids. The starch breakdown subsequently yields malate as a counterion for potassium during stomatal opening. In addition, a hexose phosphate/phosphate transport activity has also been detected in photosynthetic tissues after feeding of detached leaves with glucose (Quick et al., 1995). The chloroplasts of these leaves are photosynthetically active but contain large quantities of starch. A hexose phosphate/phosphate translocator is induced in these tissues, and as is the case for heterotrophic plastids in sink tissues, Glc6P is imported into the chloroplasts as the precursor for starch biosynthesis. Glucose feeding has obviously induced a switch in the function of the chloroplasts from carbon-exporting source organelles to carbon-importing sink organelles.

Overall, we have identified the GPT as one of the main devices to provide plastids with carbon for starch biosynthesis and/or as the substrate for the oxidative pentose phosphate pathway at the molecular level. The analysis of transgenic plants with an altered activity of the GPT will show to what extent the GPT is actually involved in these processes.

METHODS

Plant Material, cDNA Cloning, and RNA Gel Blot Analysis

Radiochemicals were purchased from Amersham-Buchler (Braunschweig, Germany) and ICN (Eschwege, Germany). Reagents and enzymes for recombinant DNA techniques were obtained from Promega (Heidelberg, Germany). The cDNA library from potato tubers was kindly provided by U. Sonnewald (Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany), and the yeast expression vector pEVP11 was provided by N. Sauer (University of Erlangen, Erlangen, Germany).

Maize (*Zea mays*), pea (*Pisum sativum*), and potato (*Solanum tuberosum*) plants were grown in the greenhouse, and cauliflower (*Brassica oleracea*) inflorescences were obtained from the local market. Isolation of poly(A)$^+$ RNA from pea leaves, cauliflower inflorescences, and maize endosperm, the subsequent syntheses of cDNAs that were cloned into λgt10, and recombinant DNA techniques were performed according to standard procedures (Ausubel et al., 1994). RNA gel blot hybridizations were performed at 42°C in a buffer containing 0.25 M sodium phosphate, pH 7.5, 0.25 M NaCl, 1 mM EDTA, 5% (w/v) polyethylene glycol, 10 μg mL^{-1} denatured salmon sperm DNA, and 25% (v/v) formamide. Filters were washed at 65°C in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.5% SDS and once in 1 × SSC and 0.5% SDS.

Isolation of the Glucose 6-Phosphate/Phosphate Translocator and Cloning of Its cDNA

Seventy grams of maize endosperm, which was prepared from kernels harvested 14 days after pollination (DAP), was ground in liquid nitrogen until the tissue was finely powdered. One hundred twenty milliliters of extraction buffer (100 mM Tricine-KOH, pH 8.0, 50 mM EDTA, 6 mM ascorbate, 0.1% BSA [w/v], 4 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) and, after thawing on ice, 150 mL of buffer A (10 mM Tricine-KOH, pH 7.8, 0.6 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride) were added. The suspension was filtered through three layers of muslin and centrifuged for 5 min at 1000*g*. The supernatant was again centrifuged for 15 min at 10,000*g*, and the resulting supernatant was centrifuged for 45 min at 44,000*g*. The pellets were resuspended in 60 mL of 1.46 M sucrose in buffer B (50 mM Tricine-KOH, pH 7.8, 3 mM $MgCl_2$, and 1 mM phenylmethylsulfonyl fluoride). Each 10 mL of this suspension was transferred into centrifuge tubes and overlaid with 12 mL of 1.0 M sucrose in buffer B followed by 13 mL of 0.73 M sucrose in buffer B and centrifuged for 12 hr at 92,000*g* (4°C). The interphase (1.0 M/0.73 M sucrose) was removed, diluted with buffer A, and washed once (40 min at 120,000*g*). The sediment was resuspended in buffer A, centrifuged again (20 min at 150,000*g*), and stored at −80°C until use.

Approximately 10 mg of the isolated membranes was suspended in 0.9 mL of buffer C (10 mM Tricine-KOH, pH 7.5, 0.2% *n*-dodecylmaltoside, and 1 mM phenylmethylsulfonyl fluoride) and solubilized by the addition of 0.24 mL of 20% *n*-dodecylmaltoside. After 2 min on ice, the suspension was diluted by the addition of 1.2 mL of buffer C and centrifuged for 3 min at 27,000*g*. The supernatant was applied to a HiTrap Q column (5 mL; Pharmacia Biotechnology) that had been equilibrated with buffer C. The pass-through of the column containing most of the glucose 6-phosphate (Glc6P)/phosphate exchange transport activity, as measured by reconstitution of the various fractions into artificial membranes (Flügge, 1992), was subjected to a chromatography on a heparin–Sepharose CL-6B column (0.2 mL; Pharmacia Biotechnology) equilibrated with buffer C. The column was washed first with buffer C (2 mL) and then eluted with 90 mM NaCl in buffer C. This eluate was subjected to SDS-PAGE (Laemmli, 1970), and two of the protein bands (31 and 30 kD) were excised from the Coomassie Brilliant Blue R 250–stained gel and digested with endoproteinase Lys-C while still in the polyacrylamide matrix. The resulting peptides were eluted and then purified by reversed-phase HPLC (Eckerskorn and Lottspeich, 1989) and sequenced in a gas phase sequencer (Eckerskorn et al., 1988).

From the 31-kD protein, sequences of two peptides were obtained: KTQVVPVQSEGAQRLK (P1) and KVAVSFTHIIK (P2). Two degenerate oligonucleotide probes were designed on the basis of the peptide sequence P1: 5′-d(AARACICARGTIGTICCIGTICA)-3′ (O1) and 5′-d(GTIGTICCIGTICARWIIGARGGIGC)-3′ (O2), where R is A or G, Y is C or T, W is A or T, and I is inosine.

A first-strand cDNA was prepared using poly(A)$^+$ RNA from maize endosperm, an oligo(dT)$_{15}$ anchor primer (rapid amplification of cDNA ends [RACE] adapter primer [RA primer; 5′-d[CCACGAGTCGACTCTAGAGCTCGGATCCTT[T]$_{15}$-3′]]), and a cDNA preamplification system, according to the instructions given by the manufacturer (Gibco-BRL Life Technologies, Eggenstein, Germany). The resulting single-stranded cDNA was the template for the RACE procedure using oligonucleotides O1 and the RA primer as forward and reverse

primers, respectively. The first reaction contained 2 μL of cDNA from the first-strand reaction, 0.1 μM RA primer, 1 μM nested RACE primer 1 (5′-d[CCACGAGTCGACTCTAG]-3′), 1 μM gene-specific primer O1, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 2 units of Taq polymerase in a final volume of 50 μL. The reaction was heated for 5 min at 94°C, and the subsequent amplification consisted of three cycles of 10 min at 37°C, 2 min at 55°C, and 1 min at 94°C, followed by 35 cycles of 1 min at 55°C, 2 min at 72°C, and 1 min at 94°C. The assay was then diluted by a factor of 100 and used for the second RACE reaction containing 2 μL of the first RACE reaction, 0.2 μM nested RACE primer 2 (5′-d[CTCTAGAGCTCGGATCC]-3′), 1 μM nested primer O2, 1.5 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, and 2 units of Taq polymerase in a final volume of 50 μL. The reaction was heated for 2.5 min at 95°C, and the subsequent amplification consisted of 30 cycles of 45 sec at 94°C, 60 sec at 45°C, and 70 sec at 72°C, followed by incubation for 5 min at 72°C. The generated DNA fragment (1200 bp) was then used for a plaque hybridization screening of cDNA libraries from maize endosperm, pea roots, potato tubers, and cauliflower inflorescences. Positive plaques were purified, and the inserts were excised and subcloned into the pBluescript KS+ (Stratagene, La Jolla, CA) for partial sequencing at both ends (Sanger et al., 1977). Full-length cDNAs were sequenced completely on both strands.

Heterologous Expression of the Glc6P/Phosphate Translocator cDNA in Yeast Cells

The DNA encoding the mature part of the Glc6P/phosphate translocator (GPT) from pea roots (Pea-GPT, amino acid residues 86 to 401 of the precursor protein) was obtained by polymerase chain reaction using the corresponding pBluescript KS+ cDNA clone as a template. As a sense primer, a synthetic oligonucleotide corresponding to the beginning of the mature part of the GPT with an additional BamHI restriction site was used (5′d[AACGGATCCGCTTATGAGGCTGATAGATCA]-3′), and the second oligonucleotide was a (reverse) T7 primer. The resulting DNA fragment was sequenced and subsequently cloned into the BamHI-HindIII–cut *Escherichia coli* expression vector pQE32 (Qiagen, Hilden, Germany), resulting in clone pQE-GPT. This clone contained a His_6-tag fused in frame to the N terminus of the mature part of the Pea-GPT protein. This construct was released by digestion with EcoRI and filled in. The DNA fragment was ligated into the blunted BamHI-cut yeast expression vector pEVP11 (Russel and Nurse, 1986) containing the *Saccharomyces cerevisiae LEU2*$^+$ gene downstream of the alcohol dehydrogenase promotor of *Schizosaccharomyces pombe.* Transformation of cells from *S. pombe*, fractionation of these cells, purification of the expressed protein on Ni^{2+}–NTA–agarose (Qiagen), and reconstitution of the transport activity were performed essentially as described by Loddenkötter et al. (1993) and Fischer et al. (1994b).

Protein Import Assay

Cauliflower bud plastids were isolated from cauliflower inflorescences essentially as described previously (Journet and Douce, 1985; Alban et al., 1988). The cloned cDNA of the GPT protein from pea roots was transcribed and translated using the TNT lysate system, according to the instructions given by the manufacturer (Promega), and ^{35}S-methionine as labeled amino acid. Protein import assays contained 250 mM sorbitol, 10 mM methionine, 25 mM potassium gluconate, 2 mM $MgSO_4$, 50 mM Hepes-KOH, pH 8.0, 0.2% BSA, radiolabeled in vitro–synthesized precursor protein (import buffer), and purified organelles equivalent to 1.7 mg of protein or 200 μg of chlorophyll, respectively, in a final volume of 300 μL. Other additions are indicated in the legend to Figure 6. The import reaction was allowed to proceed for 20 min. Isolation of envelope membranes was performed as previously described (Flügge et al., 1989). All fractions were analyzed by SDS-PAGE and fluorography.

Reconstitution of Transport Activities

Liposomes were prepared from acetone-washed soybean phospholipids (100 mg/mL) by sonication for 10 min at 4°C in 100 mM Tricine-NaOH, pH 7.5, 30 mM potassium gluconate, and 25 mM Glc6P (unless stated otherwise). Solubilized membranes from maize kernels or fractions eluted from the chromatography columns were incorporated into the liposomes by a freeze–thaw step. Reconstituted ^{32}P-phosphate transport activity was measured essentially as described by Flügge (1992) and Fischer et al. (1994b).

For the determination of substrate specificities, kinetic properties, and the stoichiometry of the Glc6P/phosphate exchange, the recombinant GPT protein that had been purified to apparent homogeneity by Ni^{2+}–nitrilotriacetic acid chromatography from *S. pombe* cells (Loddenkötter et al., 1993) was used. As eluant, a buffer containing 150 mM imidazole and 0.06% *n*-dodecylmaltoside was used. For the determination of the stoichiometry of the Glc6P/phosphate exchange reaction, this eluate was diluted twofold by the addition of liposomes (200 mg/mL) that had been prepared by sonication in the presence of 40 mM Tricine-NaOH, pH 7.4, 30 mM NaH_2PO_4, 140 mM sodium gluconate, and 100 mM potassium gluconate.

After freezing and thawing, ^{32}P-phosphate was added to half of the proteoliposomes (final specific activity of 9000 cpm/nmol). After 40 min at 20°C, the external substrate was removed from both types of liposomes by chromatography on Sephadex G-25 (Pharmacia Biotechnology), which had been equilibrated with 20 mM Tricine-NaOH, pH 7.6, 130 mM sodium gluconate, and 50 mM potassium gluconate. The eluted ^{32}P-phosphate–containing liposomes were used for the determination of the time course of ^{32}P-phosphate release, which was initiated by externally added Glc6P (1.3 mM).

The time-dependent uptake of ^{14}C-Glc6P (1.3 mM, specific activity of 6000 dpm/nmol) was monitored by using the other type of liposomes. Transport reactions were terminated by an inhibitor stop in combination with removal of the external radioactivity by passing aliquots of the liposomes over a Dowex AG-1X8 (Bio-Rad Laboratories, Munich, Germany) column, which had been preequilibrated with 180 mM sodium acetate (Flügge, 1992). The radioactivity of the eluate was determined by liquid scintillation counting.

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REFERENCES

Alban, C., Joyard, J., and Douce, R. (1988). Preparation and characterization of envelope membranes from nongreen plastids. Plant Physiol. **88,** 709–717.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215,** 403–410.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidmann, J.G., Smith, J.A., and Struhl, K., eds (1994). Current Protocols in Molecular Biology. (New York: John Wiley and Sons).

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

Borchert, S., Harborth, J., Schünemann, D., Hoferichter, P., and Heldt, H.W. (1993). Studies of the enzymatic capacities and transport properties of pea root plastids. Plant Physiol. **101,** 303–312.

Bowsher, C.G., Boulton, E.L., Rose, J., Nayagam, S., and Emes, E.J. (1992). Reductant for glutamate synthase is generated by the oxidative pentose phosphate pathway in non-photosynthetic root plastids. Plant J. **2,** 893–898.

Capaldi, R.A., and Vanderkooi, G. (1972). The low polarity of many membrane proteins. Proc. Natl. Acad. Sci. USA **69,** 930–932.

Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P., and Smith, A.M. (1996). The major form of ADP–glucose pyrophosphorylase in maize endosperm is extraplastidial. Plant Physiol. **112,** 779–785.

Eckerskorn, C., and Lottspeich, F. (1989). Internal amino acid sequence analysis of proteins separated by gel electrophoresis after tryptic digestion in polyacrylamide matrix. Chromatographia **28,** 92–94.

Eckerskorn, C., Mewes, W., Goretzki, H., and Lottspeich, F. (1988). A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. **176,** 509–519.

Entwistle, G., and ap Rees, T. (1988). Enzymatic capacities of amyloplasts from wheat (*Triticum aestivum*) endosperm. Biochem. J. **255,** 391–396.

Fischer, K., Weber, A., Brink., S., Arbinger, B., Schünemann, D., Borchert, S., Heldt, H.W., Popp, B., Benz, R., Eckerskorn, C., and Flügge, U.I. (1994a). Porins from plants: Molecular cloning and functional characterization of two new members of the porin family. J. Biol. Chem. **269,** 25754–25760.

Fischer, K., Arbinger, B., Kammerer, K., Busch, C., Brink, S., Wallmeier, H., Sauer, N., Eckerskorn, C., and Flügge, U.I. (1994b). Cloning and *in vivo* expression of functional triose phosphate/phosphate translocators from C_3- and C_4-plants: Evidence for the putative participation of specific amino acid residues in the recognition of phosphoenolpyruvate. Plant J. **5,** 215–226.

Fischer, K., Kammerer, B., Gutensohn, M., Arbinger, B., Weber, A., Häusler, R.E., and Flügge, U.I. (1997). A new class of plastidic phosphate translocators: A putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. Plant Cell **9,** 453–462.

Flügge, U.I. (1992). Reaction mechanism and asymmetric orientation of the reconstituted chloroplast phosphate translocator. Biochim. Biophys. Acta **1110,** 112–118.

Flügge, U.I. (1995). Phosphate translocation in the regulation of photosynthesis. J. Exp. Bot. **46,** 1317–1323.

Flügge, U.I., and Weber, A. (1994). A rapid method for measuring organelle-specific substrate transport in homogenates from plant tissues. Planta **194,** 181–185.

Flügge, U.I., Fischer, K., Gross, A., Sebald, W., Lottspeich, F., and Eckerskorn, C. (1989). The triose phosphate-3-phosphoglycerate-phosphate translocator from spinach chloroplasts: Nucleotide sequence of a full-length cDNA clone and import of the *in vitro* synthesized precursor protein into chloroplasts. EMBO J. **8,** 39–46.

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

Higgins, D.G., Bleasby, A.J., and Fuchs, R. (1992). CLUSTAL V: Improved software for multiple sequence alignment. Comput. Appl. Biosci. **8,** 189–191.

Hill, L.M., and Smith, A.M. (1991). Evidence that glucose 6-phosphate is imported as the substrate for starch biosynthesis by the plastids of developing pea embryos. Planta **185,** 91–96.

Hill, L.M., and Smith, A.M. (1995). Coupled movements of glucose 6-phosphate and triose phosphate through the envelopes of plastids from developing embryos of pea (*Pisum sativum* L.). J. Plant Physiol. **146,** 411–417.

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

Klösgen, R.B., Saedler, H., and Weil, J.-H. (1989). The amyloplast-targeting transit peptide of the waxy protein of maize also mediates protein transport in vitro into chloroplasts. Mol. Gen. Genet. **217,** 155–161.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Li, H., Sullivan, T.D., and Keegstra, K. (1992). Information for targeting to the chloroplastic inner envelope membrane is contained in the mature region of the maize *Bt1*-encoded protein. J. Biol. Chem. **267,** 18999–19004.

Liu, T.-T.Y., and Shannon, J.C. (1981). Measurements of metabolites associated with nonaqueously isolated starch granules from immature *Zea mays* L. endosperm. Plant Physiol. **67,** 525–529.

Loddenkötter, B., Kammerer, B., Fischer, K., and Flügge, U.I. (1993). Expression of the functional mature chloroplast triose phosphate translocator in yeast internal membranes and purification of the histidine-tagged protein by a single metal-affinity chromatography step. Proc. Natl. Acad. Sci. USA **90,** 2155–2159.

Naeem, M., Tetlow, I.J., and Emes, M.J. (1997). Starch synthesis in amyloplasts purified from developing potato tubers. Plant J. **11,** 1095–1103.

Neuhaus, H.E., and Maas, U. (1996). Unidirectional transport of orthophosphate across the envelope of isolated cauliflower-bud amyloplasts. Planta **198,** 542–548.

Neuhaus, H.E., Thom, E., Batz, O., and Scheibe, R. (1993). Purification of highly intact plastids from various heterotrophic plant tissues. Analysis of enzyme equipment and precursor dependency for starch biosynthesis. Biochem. J. **296,** 395–401.

Overlach, S., Diekmann, W., and Raschke, K. (1993). Phosphate translocator of isolated guard cell chloroplasts from *Pisum sativum* L. transports glucose-6-phosphate. Plant Physiol. **101,** 1201–1207.

Quick, W.P., and Neuhaus, H.E. (1996). Evidence for two types of phosphate translocators in sweet-pepper (*Capsicum annuum* L.) fruit chromoplasts. Biochem. J. **320,** 7–10.

Quick, W.P., Scheibe, R., and Neuhaus, H.E. (1995). Induction of a hexose-phosphate translocator activity in spinach chloroplasts. Plant Physiol. **109,** 113–121.

Russel, P., and Nurse, P. (1986). *cdc25*$^{+}$ functions as an inducer in the mitotic control of the fission yeast. Cell **45,** 145–153.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4,** 406–425.

Sanger, F., Nicklen, S., and Coulson, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74,** 5463–5467.

Schaefer, B.C. (1995). Revolutions in rapid amplification of cDNA ends: New strategies for polymerase chain reaction cloning of full-length cDNA ends. Anal. Biochem. **227,** 255–273.

Schott, K., Borchert, S., Müller-Röber, B., and Heldt, H.W. (1995). Transport of inorganic phosphate and C_3- and C_6-sugar phosphates across the envelope membranes of potato tuber amyloplasts. Planta **196,** 647–652.

Schulz, B., Frommer, W.B., Flügge, U.I., Hummel, S., Fischer, K., and Willmitzer, L. (1993). Expression of the triose phosphate translocator gene from potato is light dependent and restricted to green tissues. Mol. Gen. Genet. **238,** 357–361.

Schünemann, D., and Borchert, S. (1994). Specific transport of inorganic phosphate and C_3- and C_6-sugar-phosphates across the envelope membranes of tomato (*Lycopersicon esculentum*) leaf-chloroplasts, tomato fruit-chloroplasts and fruit-chromoplasts. Bot. Acta **107,** 461–467.

Schwarz, M., Gross, A., Steinkamp, T., Flügge, U.I., and Wagner, R. (1994). Ion channel properties of the reconstituted chloroplast triose phosphate/phosphate translocator. J. Biol. Chem. **269,** 29481–29489.

Sullivan, T.D., and Kaneko, Y. (1995). The maize *brittle1* gene encodes amyloplast membrane polypeptides. Planta **196,** 477–484.

Sullivan, T.D., Strelow, L.I., Illingworth, C.A., Phillips, R.L., and Nelson, O.E., Jr. (1991). Analysis of maize *Brittle-1* alleles and a defective *Suppressor-mutator*-induced mutable allele. Plant Cell **3,** 1337–1348.

Tetlow, I.J., Blisset, K.J., and Emes, M.J. (1994). Starch synthesis and carbohydrate oxidation in amyloplasts from developing wheat endosperm. Planta **194,** 454–460.

Tetlow, I.J., Bowsher, C.G., and Emes, M.J. (1996). Reconstitution of the hexose phosphate translocator from the envelope membranes of wheat endosperm amyloplasts. Biochem. J. **319,** 717–723.

Thorbjørnsen, T., Villand, P., Denyer, K., Olsen, O.-A., and Smith, A.M. (1996). Distinct isoforms of ADPglucose pyrophosphorylase occur inside and outside the amyloplasts in barley endosperm. Plant J. **10,** 243–250.

Tyson, R.H., and ap Rees, T. (1988). Starch synthesis by isolated amyloplasts from wheat endosperm. Planta **175,** 33–38.

Weber, A., Menzlaff, E., Arbinger, B., Gutensohn, M., Eckerskorn, C., and Flügge, U.I. (1995). The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: Molecular cloning of a transporter protein containing a 12-helix motif and expression of the functional protein in yeast cells. Biochemistry **34,** 2621–2627.

Willey, D.L., Fischer, K., Wachter, E., Link, T.A., and Flügge, U.I. (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.