Glucose 1-phosphate transport into protoplasts and chloroplasts from leaves of
Arabidopsis thaliana

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Abstract

Almost all glucosyl transfer reactions rely on glucose 1-phosphate that either immediately acts as glucosyl donor or as substrate for the synthesis of the more widely used glucose dinucleotides, ADPglucose or UDPglucose. In this communication, we have analyzed two glucose 1-phosphate related processes: the carbon flux from externally supplied glucose 1-phosphate to starch by either mesophyll protoplasts or intact chloroplasts from Arabidopsis thaliana. When intact protoplasts or chloroplasts are incubated with [U-14C]glucose 1-phosphate, starch is rapidly labelled. Incorporation into starch is unaffected by the addition of unlabeled glucose 6-phosphate or glucose indicating a selective flux from glucose 1-phosphate to starch. However, illuminated protoplasts incorporate less 14C into starch when unlabelled bicarbonate is supplied in addition to the 14C-labelled glucose 1-phosphate.

Mesophyll protoplasts incubated with [U-14C]glucose 1-phosphate incorporate 14C into the plastidial pool of adenosine diphosphoglucose. Protoplasts prepared from leaves of mutants of Arabidopsis thaliana that lack either the plastidial phosphorylase or the phosphoglucomutase isozyme incorporate 14C derived from external glucose 1-phosphate into starch but incorporation into starch is insignificant when protoplasts from a mutant possessing a highly reduced ADPglucose pyrophosphorylase activity are studied. Thus, the path of assimilatory starch biosynthesis initiated by extra-plastidial glucose 1-phosphate leads to the plastidial pool of adenosine diphosphoglucose and at this intermediate it is fused with the Calvin cycle driven route. Mutants lacking the plastidial phosphoglucomutase contain a small yet significant amount of transitory starch. The starch granule morphology is similar to that of the wild type control.
The entire metabolism of eukaryotic cells consists of distinct reaction sequences within a given compartment and the action of metabolite transporters that functionally interconnect the various compartments. Similarly, transporters located at the interface between the cells and the apoplastic space (i.e., in the cell membrane) permit an intercellular transport of metabolites and thereby integrate metabolic processes that take place in various cells. Both metabolite-related enzymes and transporters can exert a second function as they also are capable of integrating signalling paths and thereby transferring information on the metabolic status of the cellular compartments or the entire cell (Smeekens 1998; Rolland et al. 2002).

A large group of metabolite transporters is functionally related to the metabolism of sugars or sugar derivatives and some of them act within pathways that either synthesize or degrade polysaccharides. Among the plant polysaccharides, quantitatively most important are the plastidial starch and the apoplastic cell wall. Cell wall-related sugar transporters are mainly located in the endoplasmic reticulum and transport mostly nucleotide sugars (Seifert 2004).

Starch-related transporters reside in the inner envelope membrane of plastids. A glucose transporter of the envelope membrane has functionally been characterized (Weber et al. 2000). However, the quantitatively dominant product of the plastidial transitory starch degradation is maltose which is exported into the cytosol by the recently identified maltose transporter designated as MEX (maltose excess; Weise et al. 2004; Niittylä et al. 2004). Like the other transporters listed below this transporter is located in the inner chloroplast envelope membrane. As MEX deficient mutants from Arabidopsis thaliana contain exceptionally high maltose levels and exhibit a massive starch excess phenotype as well as a strong retardation in growth, MEX appears to exert an indispensable function within the starch-sucrose conversion (Niittylä et al. 2004).

Other metabolite transporters of the chloroplast envelope membranes are functionally more closely related to the reductive pentose phosphate cycle. Phosphate translocators mediate a strict counter-exchange of phosphorylated sugars (or of 3-phosphoglycerate) and orthophosphate. The triose-phosphate / phosphate translocator exports triose-phosphate (and, to some extent, 3-phosphoglycerat) from the chloroplast into the cytosol mainly during the light period (Schneider et al. 2002). The xylulose 5-phosphate / phosphate translocator has been proposed to provide the plastidial pentose phosphate pathway with reduced carbon compounds or reducing equivalents (Eicks et al. 2002; Weber 2004). Depending on concentration gradients, the phosphoenolpyruvate / phosphate translocators (strongly expressed in photosynthesis-competent cells of C4 plants) as well as the glucose 6-phosphate
phosphate translocators (prominent in heterotrophic cells) mediate the transport of the respective phosphorylated metabolite between the plastid and the cytosol. For each of the two translocators two genes have been identified that are differentially expressed in various \textit{Arabidopsis} organs (Weber 2004). Recently, evidence has been presented for the occurrence of an evolutionary conserved plastidial ADPglucose exporter (Colleoni \textit{et al.} 2010). However, the current knowledge of the metabolite transporters is far from being complete as analysis of the genome of \textit{Arabidopsis thaliana} L. revealed that approximately 140 putative metabolite transporters exist all of which are predicted to be located in the inner plastid envelope membrane (Ferro \textit{et al.} 2002; Schwacke \textit{et al.} 2003).

None of the translocators described above is able to transport another important metabolite, i.e. glucose 1-phosphate (Eicks \textit{et al.} 2002; Kammerer \textit{et al.} 1998). This glucose ester is a key intermediate in several major carbon fluxes, such as starch, sucrose and cellulose biosynthesis. In the current model of the photosynthesis-driven starch biosynthesis, the plastidial phosphoglucomutase mediates the formation of glucose 1-phosphate from glucose 6-phosphate which is directly derived from a Calvin-cycle intermediate, fructose 6-phosphate. By the action of ADPglucose pyrophosphorylase glucose 1-phosphate is then converted to ADPglucose which is the general glucosyl donor for a variety of starch synthases. The dominance of the ADPglucose-dependent path of starch biosynthesis concurs with the fact that mutants from \textit{A. thaliana} lacking the plastidial α-glucan phosphorylase isozyme (PHS1) possess essentially the same starch content as wild type plants when grown under normal conditions (Zeeman \textit{et al.} 2004). However, transgenic potato plants underexpressing both the plastidial and the cytosolic phosphoglucomutase unexpectedly possess transitory starch levels similar to those of the wild type controls. These phenotypical features are considered to be inconsistent with this model (Fernie \textit{et al.} 2002) and, therefore, the carbon fluxes towards starch appear to be more complex than often assumed.

Recently, we have shown that in heterotrophic tissues, such as potato tuber discs, glucose 1-phosphate is selectively taken up and, subsequently, enters two paths: It is metabolized via the cytosolic phosphorylase by transferring the glucosyl residue to starch-related heteroglycans (Fettke \textit{et al.} 2008). Another portion of the imported glucose 1-phosphate directly enters the amyloplasts and is converted to starch. Under normal conditions, this conversion which is mainly mediated by the plastidial phosphorylase isozyme is not the dominant pathway of reserve starch biosynthesis (Fettke \textit{et al.} 2010). However, it offers an explanation of some features of starch metabolism as observed in various mutants (see above) because this route
apparently does rely neither on the cytosolic nor the plastidial glucose 6-phosphate/glucose 1-phosphate interconversion.

Until now, the question remains whether or not this process operates also in autotrophic tissues as any evidence for a transport of glucose 1-phosphate into both mesophyll protoplasts and chloroplasts is lacking.

In this communication, we have studied the transport of glucose 1-phosphate into autotrophic cells as well as possible implications of this process for the entire transitory starch metabolism.

Results

Uptake and utilization of external glucose 1-phosphate by mesophyll protoplasts from *Arabidopsis thaliana* L.

To study the uptake of glucose 1-phosphate by intact mesophyll protoplasts, short term experiments were performed using varying concentrations of [U-14C]glucose 1-phosphate. Following an incubation of 30 sec, the protoplasts were washed and the total 14C content of the protoplasts was determined (Fig. 1A and 1B). Uptake of the 14C-labelled glucose 1-phosphate was clearly detectable at submicromolar concentrations and saturation was achieved at approximately 1 mM glucose 1-phosphate. The apparent KM was estimated to be 413 µM.

In another series of short term uptake experiments, the protoplasts were incubated with either 200 µM or 400 µM [U-14C]glucose 1-phosphate and an equal concentration of unlabelled glucose and glucose 6-phosphate, respectively. As a control, the unlabelled compound was omitted (Figure 1C). Neither the presence of glucose nor that of glucose 6-phosphate did affect the uptake of glucose 1-phosphate. Thus, the putative glucose 1-phosphate transporter is selective for both the phosphorylated glucose (as it does not react with glucose) and the anomeric position of the phosphate ester (as it does not act on glucose 6-phosphate).

In order to determine whether or not the imported glucose 1-phosphate is converted to starch (as it is the case in heterotrophic potato tuber cells; Fettke *et al.* 2010), mesophyll protoplasts from *Arabidopsis* leaves were incubated for 20 min with equimolar concentrations (20 mM each) of [U-14C]glucose 1-phosphate or [U-14C]glucose. During incubation the protoplasts were either illuminated or darkened. Following incubation, starch was isolated and the 14C content was quantified. Incubation with glucose 1-phosphate results in a more than 10fold
higher incorporation as compared to glucose (Fig. 2A). Thus, the superior efficiency of the
1-phosphate dependent labelling of starch is not unique to heterotrophic cells (cf.
Fettke et al. 2010). Light stimulates the flux from both external glucose 1-phosphate and
glucose to starch but even in darkened protoplasts the glucose 1-phosphate-dependent
labelling of starch is higher than that of glucose in illuminated protoplasts. When comparing
the incorporation into starch in illuminated and darkened protoplasts, it should be noted that
in the dark starch biosynthesis via the ADPglucose pyrophosphorylase is inhibited (Hendriks
et al. 2003) and, in addition, net starch degradation is initiated. The onset of starch
mobilization, as observed under the conditions used, was analysed by $^{14}$C-labelling of
protoplasts in the light, transfer into the dark and determination of $^{14}$C-label of starch during
the dark phase (data not shown). Therefore, in darkened protoplasts the $^{14}$C-labelling of starch
equals the total incorporation minus the release of label due to starch degradation.
For a more detailed analysis of the glucose 1-phosphate dependent incorporation into starch,
Arabidopsis mesophyll protoplasts were incubated for 5 or 10 min with one of three mixtures
each of which contained $[U^{14}$C$]$glucose 1-phosphate (20 mM each) and, in addition, one of
three unlabelled compounds (orthophosphate, glucose or glucose 6-phosphate; 10 mM each).
As a control, an aliquot of the protoplast suspension was incubated with $[U^{14}$C$]$glucose 1-
phosphate (20 mM) without adding any unlabelled compound. Protoplasts were illuminated
during incubation. The addition of any of the three unlabelled compounds increased the
glucose 1-phosphate dependent incorporation into starch but quantitatively the enhancement
differed depending upon the unlabelled compound. Glucose 6-phosphate was more effective
than glucose but orthophosphate was by far most efficient: it increased the labelling of starch
approximately 20fold as compared to the control (Fig. 2B). The stimulatory effect of
orthophosphate clearly excludes the possibility that the glucose 1-phosphate dependent
labelling of starch is due to a direct glucosyl transfer to the starch granules that is catalyzed by
a phosphorylase and takes place outside the intact protoplasts. In principle, any unnoticed
breakage of protoplasts could release both native starch granules and phosphorylase activity.
However, the phosphorylase-mediated glucosyl transfer to starch granules that occurs outside
the protoplasts would be inhibited by orthophosphate. As a control, protoplasts were
mechanically broken and the conversion of glucose 1-phosphate to starch within 10 min was
monitored to be less than 1 % as compared to that of the intact protoplasts (data not shown).
Furthermore, this type of starch labelling is not expected to be stimulated by light (Fig. 2B;
see also Fig. 3A).
During incubation, the glucose 1-phosphate dependent carbon flux to starch is far from being constant as most of the $^{14}$C is incorporated into starch during the first 5 min of incubation and the extension to 10 min results in only a very small increment of the labelling (Fig. 2B). At the molecular level, this result is difficult to explain as the entire flux is based on a series of reactions and includes a variety of components several of which could be limiting factors or steps but are difficult to estimate. Of special relevance appears to be a possible limitation exerted by the first step, i.e. the glucose 1-phosphate import, by the cytosolic orthophosphate level. If so, the simultaneous addition of orthophosphate and glucose 1-phosphate would lead to a higher cytosolic orthophosphate concentration which then would stimulate the import of the glucose phosphate.

In order to analyse a possible interdependence of the transport of orthophosphate and of glucose 1-phosphate more directly, two additional labelling experiments were performed. It should be noted that in these experiments either the total label inside the protoplasts (Fig. 2C) or the label released from the protoplasts into the medium (Fig. 2D) was monitored. In the first experiment, protoplasts were incubated in mixtures containing 10 mM [$^{33}$P]orthophosphate and, in addition, either unlabelled glucose 1-phosphate or unlabelled glucose (20 mM each). As a control, the incubation medium contained only [$^{33}$P]orthophosphate. Following the incubation for 5 min in the light, the protoplasts were carefully washed to remove external $^{33}$P-label and the amount of $^{33}$P inside the protoplasts was monitored (Fig. 2C). The addition of unlabelled glucose 1-phosphate decreased the $^{33}$P-label inside the protoplasts as compared to the control. By contrast, equimolar external glucose did not affect the $^{33}$P content of the protoplasts. This result is consistent with the assumption that external glucose 1-phosphate is imported into the cell via a glucose 1-phosphate /orthophosphate exchange that takes place at the plasmalemma and thereby diminishes the orthophosphate-dependent prelabelling of the protoplasts.

In the second experiment, protoplasts were prelabelled by incubation with [$^{33}$P]orthophosphate for 10 min in the light. Subsequently, the residual external orthophosphate was carefully removed by repeated washing steps and then the protoplast suspension was divided into two equal parts that were incubated for 5 min in the light either in the presence or in the absence (control) of 20 mM glucose 1-phosphate. Finally, the protoplasts were pelleted by centrifugation and the $^{33}$P content in the supernatant was monitored (Fig. 2D). External glucose 1-phosphate increased the release of orthophosphate into the medium more than twofold as compared to the control.
In summary, both labelling experiments clearly indicate that the mesophyll protoplasts import external glucose 1-phosphate by an antiport mechanism which utilizes orthophosphate as counter-ion.

Utilization of external glucose 1-phosphate by isolated *Arabidopsis* chloroplasts

The results shown in Fig. 1 imply that glucose 1-phosphate is taken up by the cells and is converted to starch. This raises the question whether or not glucose 1-phosphate directly enter the chloroplasts were the starch synthesis takes place. Until now, glucose 1-phosphate has not been reported to be imported into chloroplasts but indirect evidence for an uptake by non-green plastids has been published (potato tubers: Kosegarten and Mengel 1994; Naeem *et al.* 1997; wheat: Tetlow *et al.* 1996; soy bean: Coates and ap Rees, 1994). To answer the question mentioned above, chloroplasts isolated from *Arabidopsis* leaves were incubated with [U-14C]glucose 1-phosphate. As a control, an aliquot of the same chloroplast preparation was mechanically broken by using a potter and, subsequently, the homogenate was then treated identically. Isolated chloroplasts are able to take up glucose 1-phosphate and to convert it into starch at a relatively high rate. This process requires, however, the intactness of the organelles (Figure 3A). Thus, both the uptake of glucose 1-phosphate and the flux towards starch are functional in isolated intact chloroplasts.

In an additional experiment, chloroplasts were incubated with a mixture of [U-14C]glucose 1-phosphate and (unlabelled) orthophosphate or with [U-14C]glucose 6-phosphate. As a control, chloroplasts were incubated only with [U-14C]glucose 1-phosphate. For the three incubation mixtures, the incorporation of 14C into starch was monitored (Figure 3B). In the presence of both [U-14C]glucose 1-phosphate and orthophosphate, incorporation into starch is decreased (but still exceeds that observed with glucose 6-phosphate) suggesting a glucose 1-phosphate / orthophosphate antiport that is functional at the envelope membranes of the chloroplasts.

These data are further supported by the fact that simultaneous incubation of chloroplasts with 33P-labelled orthophosphate and unlabelled glucose 1-phosphate results in a decreased uptake of orthophosphate as compared to the incubation only with 33P-labelled orthophosphate (data not shown). This result is expected if the external glucose 1-phosphate is imported via an exchange with internal orthophosphate. As the incubation of isolated chloroplast with equimolar [U-14C]glucose 6-phosphate results in a minor labelling of starch (Fig. 3B), import and plastidial metabolism of the two externally supplied glucose phosphate esters differ.

In another series of experiments isolated chloroplasts were incubated with either [U-14C]glucose 1-phosphate only or together with unlabelled glucose 6-phosphate or glucose
(16.67 mM each). $^{14}$C incorporation into starch was monitored after 10 min or 20 min of incubation. Neither unlabelled glucose 6-phosphate nor free glucose did significantly affect the incorporation of $^{14}$C into starch indicating a selective import of the anomeric glucose ester into the chloroplast (Fig. 3C).

In summary, all the data shown in Fig. 3 indicate that mesophyll cells from leaves are capable of importing glucose 1-phosphate from the cytosol into the chloroplast stroma.

**Contribution of the plastidial $\alpha$-glucan phosphorylase isozyme (AtPHS1) to the conversion of glucose 1-phosphate to starch**

Following the uptake into the chloroplasts, glucose 1-phosphate could be further metabolized by two distinct starch synthesizing pathways: First, in a single reaction (that is mediated by the plastidial $\alpha$-glucan phosphorylase isozyme, AtPHS1) glucosyl residues could be transferred directly to acceptor sites at the surface of native starch granules. Alternatively, it could undergo a more complex sequence: first the conversion of glucose 1-phosphate to ADPglucose via ADPglucose pyrophosphorylase (Lin *et al.* 1988) and, subsequently, the glucosyl transfer from ADPglucose to glucans of the starch granule that is catalyzed by at least five ADPglucose-dependent starch synthase isozymes.

In order to test the existence and/or relevance of the PHS1-dependent path, *Arabidopsis* insertion mutants were used that are deficient in the plastidial phosphorylase isozyme (AtPHS1; Zeeman *et al.* 2004). The phosphorylase pattern from *Arabidopsis* wild type leaves consists of four bands of activity all which strictly depend on glucose 1-phosphate (Figure 4A). In a glycogen-containing separation gel, the two slowly moving bands represent the cytosolic phosphorylase isoform (AtPHS2) that exists in two states differing in the apparent affinity toward the immobilized polyglucan (Fettke *et al.* 2005b). Similarly, the plastidial phosphorylase isoform (AtPHS1) occurs in two distinct but faster moving bands (Fig. 4A). In knock-out mutants lacking the plastidial phosphorylase these two bands are undetectable suggesting that they are products of the same gene. The structural and functional implications of the heterogeneity of AtPHS1 and AtPHS2 are unknown.

As compared to the wild-type control, mesophyll protoplasts from AtPHS1-deficient lines did not differ in the glucose 1-phosphate dependent incorporation into starch (Fig. 4B). Therefore, in mesophyll cells the direct glucosyl transfer to starch, as mediated by AtPHS1, appear to be of no or minor relevance. By contrast, in potato tuber discs the conversion of glucose 1-phosphate into starch did reflect the level of the plastidial phosphorylase activity (Fettke *et al.* 2010).
ADPglucose-dependent $^{14}$C-incorporation into starch

As the plastidial glucose 1-phosphate pool is not noticeably used for any AtPHS1-mediated $^{14}$C-labelling of starch (Fig. 4B), we tested whether or not the conversion of glucose 1-phosphate to starch does include both the action of ADPglucose pyrophosphorylase and the incorporation into the plastidial ADPglucose pool. If so, the activity of the Calvin cycle that also feeds into this starch forming path is expected to affect the conversion of the externally supplied glucose 1-phosphate towards starch. In order to test this possibility, we incubated protoplasts with 10mM [U-$^{14}$C]glucose 1-phosphate in either the presence or the absence of 5mM unlabelled HCO$_3^-$ $^{14}$C-labelling of starch is significantly reduced by the addition of unlabelled hydrogen carbonate (Table I). These results strongly suggest that the imported glucose 1-phosphate and intermediates of the Calvin cycle enter the plastidial ADPglucose pool and, subsequently, utilize the same reactions that transfer glucosyl residues to starch. If so, incubation of protoplasts with [U-$^{14}$C]glucose 1-phosphate should result in a $^{14}$C-labelling of ADPglucose. To test this prediction, we incubated protoplasts derived from wild-type leaves with [U-$^{14}$C]glucose 1-phosphate for 10 or 20 min in the dark or in the light. At intervals, aliquots of the incubation mixture were withdrawn and metabolic processes were terminated by the addition of ethanol (final concentration 50% [v/v]) followed by heating. By this treatment, proteins were denatured and metabolites were extracted. Subsequently, the extracts were lyophilized, dissolved in water and were then reacted with a mixture of native potato tuber starch and recombinant starch synthase III derived from A. thaliana. Following careful washing, the $^{14}$C incorporation into native starch granules was monitored (Table IIa). Due to the selectivity of the recombinant starch synthase, this method permits the quantification of the $^{14}$C content of ADPglucose even in the presence of a large excess of [U-$^{14}$C]glucose 1-phosphate. In addition, aliquots of the protoplast suspension were used to monitor the incorporation into starch by the intact protoplasts during incubation with [U-$^{14}$C]glucose 1-phosphate (Table IIb).

During illumination of protoplasts, import of [U-$^{14}$C]glucose 1-phosphate results in $^{14}$C incorporation into both starch and ADPglucose. Both processes require intactness of the protoplasts. In the dark, almost no starch is labelled and, in addition, less $^{14}$C-ADPglucose is formed (Table IIa and b). This result is not unexpected as the synthesis of ADPglucose via ADPglucose pyrophosphorylase has been reported to be light dependent (Hendriks et al. 2003).
Starch synthesis in the Arabidopsis pgm1 mutant

The pgm1 mutant from A. thaliana that lacks a functional plastidial phosphoglucomutase is incapable to perform a Calvin-cycle driven biosynthesis of assimilatory starch (Caspar et al. 1985). For a long time this mutant has been considered to lack any leaf starch. However, some recently published data indicate that it does indeed contain starch although in very small quantities (e.g. Niittylä et al. 2004; Munoz et al. 2005). This implies that, although to a far lower extent, assimilatory starch can be formed by an additional, yet unknown path which does not include the plastidial conversion of the Calvin cycle-derived glucose 6-phosphate to glucose 1-phosphate. The import of glucose 1-phosphate into the chloroplast, as analysed in this study, could be an essential step within this path.

To test this assumption, we prepared mesophyll protoplasts from the pgm1 mutant and incubated the protoplasts with [U-14C]glucose 1-phosphate during illumination. For comparison, protoplasts were isolated from leaves of two types of A. thaliana plants that had been grown under essentially the same conditions and had been treated identically: wild type plants and a mutant having a strongly reduced level of the ADPglucose pyrophosphorylase activity (adg1; Lin et al. 1988). At intervals, aliquots of the three protoplast preparations were withdrawn and, following the extraction of starch, the 14C content was monitored. In the pgm1 mutant 14C-label was clearly detectable in the starch fraction and the 14C incorporation proceeded with time (Fig. 5A). Thus, 14C derived from the externally supplied glucose 1-phosphate was taken up by the protoplasts, imported into the chloroplast and was then incorporated into starch even in the absence of a functional pPGM. However, labelling of starch was lower as compared to the wild type control. By contrast, the mutant from A. thaliana having a lower ADPglucose phosphorylase activity incorporated very little 14C into starch and labelling was essentially unchanged during 5 to 20 min incubation (Fig. 5A). Thus, the residual ADPglucose pyrophosphorylase activity of this mutant is insufficient to sustain the carbon flux from externally supplied glucose 1-phosphate towards starch.

Regarding the pgm1 mutant, the in vivo flux from glucose 1-phosphate into the plastids is minor and, therefore, unable to permit normal starch accumulation. At the end of the light period, we monitored the leaf starch content to be 0.09 ± 0.006 mg glucose g FW⁻¹ whereas the Col-0 wild type plants, grown under the same conditions, contained 6.843 ± 0.427 mg glucose g FW⁻¹ (n = 4). Despite the low starch content of the pgm1 mutant, we were able to isolate native leaf starch granules (Fig.5C). Based SEM examination, the morphology of the mutant-derived starch particles is similar to those obtained from wild type leaves (Fig. 5B; see also Streb et al. 2009).
For several reasons, the relatively small contribution of the glucose 6-phosphate independent flux to the total starch biosynthesis is not surprising: Firstly, in the cytosol glucose 1-phosphate is used in various (and, possibly, competing) reactions among which the formation of UDPglucose is most prominent. Subsequently, UDPglucose is used for the biosynthesis of sucrose and cell wall polysaccharides. Secondly, the total cellular content of glucose 1-phosphate is very low and undergoes only moderate changes throughout the light-dark cycle (e.g. Schneider et al. 2002). Finally, provided an appropriate gradient is given an efficient glucose 1-phosphate / phosphate antiporter located in the chloroplast envelope membrane(s), will even lower the plastidial glucose 1-phosphate pool if mediating a bidirectional transport. Thereby, the transporter will diminish the contribution of the Calvin-cycle-independent path of starch biosynthesis.

Discussion

In this communication, we provide evidence that photosynthesis-competent mesophyll cells from leaves of A. thaliana are capable of utilizing extracellular glucose 1-phosphate. Following uptake, glucose 1-phosphate passes the plastidial envelope membranes and, finally, the hexosyl residue is converted to starch.

Most of the experiments described here have been performed by using isolated mesophyll protoplasts that, to some extent, are a non-physiological system possessing altered carbon fluxes such as the re-synthesis of cell wall materials that may be favoured over other biosynthetic paths. However, for the present study both isolation and treatment of protoplasts had been carefully optimized. Under the conditions used, more than 85 % of the protoplasts remained functional over a period of 24 h (data not shown). Nevertheless, control experiments were included ensuring that the biochemical processes studied are restricted to intact protoplasts. When using these precautions, functional protoplasts offer some unique advantages as, in short term experiments, they permit a quantitative analysis of the uptake of metabolites. For putative transporters, apparent $K_m$ and $V_{max}$ values can be determined which is impossible if more physiological systems, such as leaf tissues, are applied. As an example, due to both the geometry of leaf discs and the structural complexity of the apoplastic space the uptake of a given metabolite into a cell is unavoidably superimposed (and largely affected) by the diffusion of that compound to the vicinity of the respective cell and, therefore, neither effective extracellular concentrations of the metabolite nor uptake rates can be determined.
Based on the experiments described above we propose a schema of transitory starch biosynthesis outlined in Fig. 6.

The transfer of glucosyl residues to non-reducing ends of native starch granules is mediated by various isoforms of starch synthase (EC 2.4.1.21) that all utilize ADPglucose as donor. The glucosyl donor is formed by the action of the ADPglucose pyrophosphorylase according to the equation: $\text{ATP + glucose 1-phosphate } \leftrightarrow \text{ADPglucose + PP}_i$.

However, two distinct routes lead to the plastidial pool of glucose 1-phosphate. One is well established and driven by the Calvin cycle intermediate, fructose 6-phosphate which, in a two step reaction, is converted to glucose 6-phosphate (mediated by the plastidial hexose phosphate isomerise (pPGI) and, subsequently, to glucose 1-phosphate (mediated by the plastidial phosphoglucomutase, pPGM). In *A. thaliana* this route is by far dominant and, therefore, mutants lacking either functional pPGI or pPGM are largely (but not completely) impaired in the biosynthesis of assimilatory starch. A second path consists of the direct import of glucose 1-phosphate into the chloroplast where it joins the pool of the hexosyl phosphate used by ADPglucose pyrophosphorylase. However, the import of glucose 1-phosphate cannot compensate the biosynthetic route derived from fructose 6-phosphate and, therefore, the amount of native starch granules that is found in pPGM-deficient plants accounts for only slightly more than 1 % as compared to the wild type (see above).

To some extent, the data reported here concur with the metabolism of glucose 1-phosphate as described for heterotrophic tissues, such as potato tubers (Fettke *et al.* 2008; Fettke *et al.* 2010). Tuber parenchyma cells are capable of importing glucose 1-phosphate and incorporate the glucosyl residues into starch. Evidence has been presented that this process includes the action of two transporters located at the plasmalemma and the amyloplast envelope membranes, respectively (Fettke *et al.* 2010). In addition, the imported glucose 1-phosphate has been shown to be incorporated, via the cytosolic phosphorylase isozyme, into cytosolic heteroglycans (Fettke *et al.* 2008). However, this reaction seems to be of minor relevance for the carbon flux directed to starch as a reduced activity of the cytosolic phosphorylase did not affect the [U-14C]glucose 1-phosphate dependent labelling of starch. Nevertheless, this pathway could be involved in buffering the cytosolic glucose 1-phosphate pool.

Following the import of glucose 1-phosphate into the amyloplasts, the subsequent incorporation into starch is mainly mediated by the plastidial phosphorylase isozyme (Pho1; Fettke *et al.* 2010). By contrast, in mesophyll cells glucose 1-phosphate imported into the chloroplast leads to the formation of ADPglucose which then acts as glucosyl donor for starch
synthases (Tab. II). These results are in agreement with the conclusion that in *Arabidopsis thaliana* the plastidial phosphorylase is not essential for starch metabolism under normal growth conditions (Zeeman *et al.* 2004).

In potato tubers the Pho1-dependent path seems to be of minor relevance under normal *in vivo* conditions. Potato mutants lacking a functional plastidial phosphoglucosomutase accumulate by far less reserve starch as compared to wild type controls (Taubberger *et al.* 2000). Similarly, *Arabidopsis* plants (and potato leaves as well) lacking a functional plastidial phosphoglucosomutase (Caspar *et al.* 1985) contain less starch than wild type leaves. These mutants are capable of forming small amounts of leaf starch by a pathway that is not directly linked to the functional Calvin cycle (Fig. 6; see also Streb *et al.* 2009). However, *Solanum tuberosum* lines possessing an antisense repression of both the cytosolic and the plastidial phosphoglucosomutase unexpectedly exhibit a phenotype similar to the wild type (Fernie *et al.* 2002). Similarly, the sta5-1 mutant from *Chlamydomonas reinhardtii* that is reported to lack the plastidial phosphoglucosomutase accumulates 4 to 12% of the normal starch amounts (Van den Koornhuyse *et al.* 1996). In all these cases an import of glucose 1-phosphate into the plastid can, at least partially, compensate the blocked glucose 6-phosphate/glucose 1-phosphate conversion inside the plastid and, therefore, explains the described phenotypes. For several reasons, it is not unexpected that the flux outlined above often permits only a partial restoration of starch biosynthesis. The interconversion of the two glucose monophosphates, as mediated by the phosphoglucosomutase, has a thermodynamical equilibrium of close to unity. However, the quantification of the two glucose esters in several tissues indicates that the glucose 6-phosphate levels exceed those of the glucose 1-phosphate and, therefore, strongly suggest that *in vivo* the phosphoglucosomutase-mediated reaction is not at equilibrium (Tarnowsky *et al.* 1964; Alpers 1968; Tetlow *et al.* 1998). Thus, the import of glucose 1-phosphate into the plastids appears to be limited by the cytosolic concentration of the substrate. Interestingly, in mutants lacking a functional phosphoglucosomutase the glucose 6-phosphate content is increased ten-fold but that of glucose 1-phosphate is only slightly higher as compared to the wild type controls (Kofler *et al.* 2000). Furthermore, recently published data strongly indicate that the expression of metabolite transporters located at the chloroplast envelope is affected by alterations in the central carbon metabolism. In pPGM lacking mutants of *A. thaliana*, the plastidial transport activity for both glucose 6-phosphate and phosphoglycerate is significantly increased (Kunz *et al.* 2010). Possibly, the flux from the plastidial glucose 6-phosphate pool into the cytosol is enhanced which results in a faster formation of glucose 1-phosphate by the cytosolic phosphoglucosomutase activity. Furthermore,
alterations in the central carbon metabolism affect the expression of other transporters of the chloroplast envelope as well. In wild type leaves, expression of glucose 6-phosphate transporter is weak (Niewiadomski et al. 2005). However, for the plastidial glucose-6-phosphate/phosphate translocator mutants both a strongly increased expression of a second isoform of this transporter (GPT2) and an enhanced transport rate have been reported (Kunz et al. 2010).

In order to analyse the flexibility of the primary metabolism, two double mutants from A. thaliana have been generated in our lab that lack both, the plastidial phosphorylase (PHS1) and the ADPglucose pyrophosphorylase or AtPHS1 plus pPGM. Phenotypical analyses of these mutants are in progress.

The uptake of glucose 1-phosphate by isolated chloroplasts from leaves of A. thaliana, as shown in this study, appears to contradict a previous study performed with spinach chloroplasts. In this study the conclusion was reached that externally supplied glucose 1-phosphate does not permit any significant starch biosynthesis (Quick et al. 1995). However, in the study cited chloroplasts were isolated from spinach leaves that had been fed for several days with glucose. It remains to be clarified whether or not the long-term feeding of the spinach leaves with glucose affects the uptake and/or intrachloroplastidial metabolism of glucose 1-phosphate. In our experiments the glucose 1-phosphate-dependent incorporation into starch occurs at relatively high rate that exceeds the rate of the photosynthesis-dependent starch synthesis (data not shown).

The glucose 1-phosphate dependent starch labelling in mesophyll protoplasts is much higher than that observed during incubation with glucose (Fig. 2). Surprisingly, simultaneous incubation of the protoplasts with [U-14C]glucose 1-phosphate and orthophosphate results in a strongly increased labelling of starch. This enhancement suggests an efficient limitation of the glucose 1-phosphate import by the cytosolic orthophosphate level. However, the simultaneous incubation with [U-14C]glucose 1-phosphate and glucose or glucose 6-phosphate also results in an enhanced labelling of starch. Currently, this effect is difficult to explain. It should, however, be taken into consideration that metabolic paths are often superimposed by sugar-mediated signalling effects that alter carbon fluxes (see above).

Our results clearly demonstrate that glucose 1-phosphate is taken up and, subsequently, is very efficiently metabolized by mesophyll protoplasts from A. thaliana. The direct interconnection between the cytosolic and plastidial glucose 1-phosphate pools suggests so far unnoticed intracellular carbon fluxes towards the plastidial starch that increase the flexibility of the plant primary metabolism. In planta under normal conditions these pathway seems to
be of minor relevance but under some conditions (such as lower temperatures; Sato et al. 2008) or distinct mutations it enable the plant to balance those particular situations. It remains to be clarified whether or not extracellular glucose 1-phosphate and its fast uptake by autotrophic cells will also constitute an efficient intercellular carbon flux.

Materials and Methods

Plant Material
Arabidopsis thaliana L. plants (Col-0, Ws, pgm1, Atphs1-1, Atphs1-2) were grown in growth chambers using either 12 h light [20°C] / 12 h dark [16°C] cycle or 14h light [22°C]/ 10h dark [17°C]. Throughout the light / dark cycles, relative humidity was 60%.

Protoplast preparation
Mesophyll protoplasts were prepared from Arabidopsis plants grown for 3-4 weeks in soil. Leaves (3-4 g each) that had been washed with water were transferred into 400 mM mannitol dissolved in water. Following slicing (approximately 4mm thickness) the leaf material was incubated for 3-3.5 h at 25°C under continuous gentle shaking in a incubation medium consisting of 5 mM MES-KOH pH 5.6, 400 mM mannitol, 8 mM CaCl₂, 1 % [w/v] cellulase Onozuka R-10 (no. 16419; from Trichoderma vivide; Serva, Heidelberg, Germany), and 0.25 % [w/v] macerozyme R-10 (no. 28302; from Rhizopus sp.; Serva). Following incubation, protoplasts were separated from the residual leaf material by filtration through a nylon mesh (350µm). In the filtrate the protoplasts were pelleted by centrifugation (90 g for 12 min; RT). After resuspending in the resuspension medium (5 mM MES-KOH pH 5.6, 400 mM mannitol, 15 mM MgCl₂) the protoplasts were washed three times with the same medium and centrifugation (as above). Subsequently, the number of protoplasts was determined by using a Thoma counting chamber (Thoma, Marienfeld, Germany) and the protoplast suspension was diluted to 3x10⁵ cells per mL (Gandhir and Khurana 2001) by using the resuspension medium. The proportion of intact protoplast was determined by treatment with fluoresceine diacetate (Larkin, 1976). For all experiments, protoplast preparations with an intactness of more than 90% were used.

Labelling experiments
a) ¹⁴C incorporation into starch. The labelling experiments were performed by incubating 15 mL of the protoplast suspension with [U⁻¹⁴C]glucose 1-phosphate or [U⁻¹⁴C]glucose (final
concentration of 20 mM each; containing 111 kBq or 46.25 kBq; GE Healthcare, Freiburg, Germany). Alternatively, the protoplasts (7.5 mL each) were incubated with [U-\(^{14}\)C]glucose 1-phosphate (in each case: final concentration 20 mM; containing 37 kBq), [U-\(^{14}\)C]glucose 1-phosphate plus unlabelled 10 mM glucose, [U-\(^{14}\)C]glucose 1-phosphate plus unlabelled 10 mM glucose 6-phosphate, [U-\(^{14}\)C]glucose 1-phosphate plus 10 mM unlabelled orthophosphate. At intervals aliquots of the protoplast suspension were withdrawn, immediately frozen in liquid nitrogen and stored at -80°C until use. Following thawing and centrifugation (10,000 g for 12 min; 4°C) the pellets were resolved in 20 % [v/v] ethanol and centrifuged (as above). The resulting pellets were resuspended in 80 % [v/v] ethanol and decolourized at 70°C. The samples were centrifuged (as above) and the pellets were collected. Following the addition of 1 mL water each, the suspensions were mixed and centrifuged (as above). Subsequently, 500 µL 200 mM KOH was added to each pellet and the mixtures were incubated for 1h at 95°C. Following neutralisation with 1 M acetic acid, the samples were centrifuged (as above) and the \(^{14}\)C content of the supernatants was monitored by using a liquid scintillation counter (Beckman Coulter, Krefeld, Germany). As revealed by treatment with amyloglucosidase, more than 98% of the \(^{14}\)C-labelled material solubilized by KOH consists of \(\alpha\)-polyglucans and, therefore, this fraction is designated as starch.

**b) Exchange experiments.**

The orthophosphate related exchange was analyzed by two types of experiments. Throughout both types of experiments, the protoplasts were illuminated (80 µmol s\(^{-1}\)m\(^{-2}\); room temperature). Type 1: Resuspended protoplasts (each 15 mL) were incubated for 5 min in the presence of 10 mM (185 kBq) \([^{33}\text{P}]\)orthophosphate or in a mixture containing 10 mM \((185\text{kBq})\) \([^{33}\text{P}]\)orthophosphate and 20 mM unlabelled glucose 1-phosphate. The protoplasts were then repeatedly washed with resuspension medium until the total radioactivity in the washing solution was below 100 dpm. The pelleted protoplasts were then resuspended in 1 mL water and the radioactivity was monitored using a liquid scintillation counter. Type 2: Protoplasts (30 mL) were incubated for 10 min with 1.85 MBq \([^{33}\text{P}]\)orthophosphate (specific activity 3000 Ci/mmol) and, subsequently, were washed three times with resuspension medium. The protoplast suspension was then divided in two equal parts. One part was incubated for 5 min in the presence of 20 mM unlabelled glucose 1-phosphate. The other part (control) was incubated for 5 min in the absence of glucose 1-phosphate. Following incubation the protoplasts were pelleted by centrifugation (90 g for 12 min) and in the supernatant the \(^{33}\)P content was determined using a liquid scintillation counter.
c) Short term uptake experiments

For short time uptake experiments protoplasts (4 mL) were incubated with either glucose 1-phosphate, glucose 1-phosphate and glucose, or glucose 1-phosphate and glucose 6-phosphate (concentration as indicated and 37 kBq [U-14C]glucose 1-phosphate was added) for 30 s at room temperature and illumination. The protoplasts were then immediately centrifuged (90 g for 3 min, 4°C) and the supernatant was discarded. The pelleted protoplasts were resuspended in 8 mL resuspension medium and centrifuged again (90 g for 3 min, 4°C). This washing step was repeated two times. Finally 1 mL water was added to the the pelleted protoplasts and the total 14C content was quantified by liquid scintillation counting.

d) Quantification of 14C-ADPglucose

Protoplasts were incubated in a resuspension medium containing 1mM unlabelled glucose 1-phosphate and 74 kBq [U-14C]glucose 1-phosphate in the light or in the dark. In the later case, protoplasts were pre-darkened for 1h. After 10 or 20min incubation, the entire suspension was immediately frozen in liquid nitrogen. Following addition of ethanol (final concentration 50% [v/v]), the suspension was heated (10min at 90°C). Following cooling and centrifugation (10,000 g for 10min), each pellet was used for isolation of starch and monitoring of the 14C content (see above). Each supernatant (containing soluble metabolites plus externally supplied compounds) was lyophilised and was then dissolved in 3.5 mL water. 1.5 mL of each solution was added to the reaction buffer (as final concentration, 40 mM Tricin pH 8.0, 1.6 mM EDTA, 20 mM potassium acetat, 75 mM citrate, and 13 mg native potato tuber starch). The glucosyl transfer from ADPglucose to the native starch granules was started by addition of 17 µg recombinant starch synthase III (AtSSIII) and was continued for 30 min at 30°C. Subsequently 25µmol unlabelled ADPglucose and 5µg AtSSIII were added and the mixture was incubated for an additional 1h. As controls, either the AtSSIII was omitted (negative control) or, alternatively, 8.3 kBq [U-14C]ADPglucose (positive control) was added during incubation. Finally the samples were centrifuged (14,000 g for 2 min) and the pelleted starch was washed six times by resuspending in 1mL water each and centrifugation (as above). In the pelleted starch the total 14C content was quantified by liquid scintillation counting.

Chloroplast isolation and labelling

Chloroplasts were prepared according to Shi et al. (2000) with minor modifications. Approximately 2 g Arabidopsis leaves were harvested in the beginning of the light period,
freed of mid veins, cut into small slices and incubated in 50 mL precooled isolation buffer
containing 20 mM Tricine-NaOH pH 8.4, 300 mM sorbitol, 10 mM EDTA, 10 mM KCl, 0.25%
[w/v] BSA, 5 mM Na-ascorbate, and 5 mM DTE. The slices were homogenized two times
for three seconds each using a Waring Blender and the resulting homogenate was filtered
through two layers Miracloth (Calbiochem-Novabiochem Corporation, LaJolla, CA, USA).
The filtrate was centrifuged (750 x g for 1 min; 4°C) and the pelleted chloroplasts were
resuspended in precooled isolation buffer (as above). The isolated chloroplasts were incubated
with [U-14C]glucose 1-phosphate (final concentration of 16.67 mM; containing 74 kBq; final
volume 4 mL). As controls the isolated chloroplasts were broken by using a potter and the
homogenate was incubated under otherwise identical conditions. Alternatively, the
chloroplasts were incubated with [U-14C]glucose 1-phosphate (16.67 mM; containing 74 kBq;
final volume 4 mL) or [U-14C]glucose 1-phosphate (16.67 mM; containing 74 kBq; final
volume 4 mL) plus unlabelled 6.67 mM orthophosphate or glucose 6-phosphate (16.67 mM;
containing 74 kBq; final volume 4 mL) or [U-14C]glucose 1-phosphate (16.67 mM;
containing 74 kBq; final volume 4 mL) plus unlabelled glucose (16.67 mM or [U-14C]glucose
1-phosphate (16.67 mM; containing 74 kBq; final volume 4 mL) plus unlabelled glucose 6-
phosphate (16.67 mM). Throughout the experiments, the chloroplasts were illuminated (80
µmol s^-1 m^-2; room temperature). At intervals, aliquots of the suspension were withdrawn and
immediately frozen in liquid nitrogen. Following thawing, the samples were centrifuged
(10,000 x g for 10 min; 4°C). The pellets were resuspended in 20 % [v/v] ethanol and were
centrifuged again (as above). The pellets were decolourized in 80 % [v/v] ethanol at 70°C (20
min). After the centrifugation (as above) the pellets were washed three times with 80 % [v/v]
ethanol. The resulting pellets were treated with 200 mM KOH for 1h at 95°C. Following
neutralisation with 1 M acetic acid, the samples were centrifuged and the 14C content in the
supernatant (i.e. starch; see above) was monitored.

Isolation of native starch granules
During the light period leaf material (35 g) from the pgm1 mutant plants was harvested and
was immediately frozen in liquid nitrogen. Following homogenization using a mortar, native
starch was isolated according to Ritte et al. (2000) with minor modification. Following a
passage through a percoll cushion (4,000 g for 5 min; 4 °C) the pelleted starch was washed
twice with extraction buffer (see Ritte et al. 2000) and resuspended in 200 µL of the same
buffer. Contaminating compounds were removed by adding an equal volume of
phenol:chloroform (v/v) mixture (1:1) to the starch suspension and centrifugation (9,000 x g
for 1 min. The aqueous phase containing the starch particles was mixed with 1 mL of
chloroform and centrifuged (as above). Subsequently the upper phase was centrifuged for 5
min at 13,000 x g and the pellet starch was collected.

Potato tuber starch was isolated according to Ritte \textit{et al.} (2000). Potato tuber tissue (15-20 g)
was mixed with 50 mL buffer (20 mM Hepes/KOH, pH 7.5 and 0.05% Triton-X-100) and
was homogenized for 20 s using a Waring blender. The homogenate was passed through
nylon net (100 µm mesh width). In the filtrate, starch granules were allowed to settle for 20
min and the supernatant was discarded. The starch pellet was washed 5 times with water and
finally was lyophilized. SEM analyses of the native starch granules were performed after
coating with gold with a Quanta apparatus (Philips).

\textbf{Extraction of buffer-soluble proteins}

Leaf material was frozen in liquid nitrogen and homogenized using a mortar. Per 1 g of fresh
weight 1 mL precooled buffer A (100 mM HEPES-NaOH pH 7.5, 1 mM EDTA, 2 mM
dithioerythritol [DTE], 10 % [w/v] glycerol, and 0.5 mM phenylmethylsulfonyl fluoride) was
added. All subsequent steps were performed at 4°C. The resulting homogenates were
centrifuged (20,000 x g for 12 min) and the supernatants were passed through a nylon net
(range 60-100 µm). The filtrates were used for protein quantification and for native PAGE.

\textbf{Quantification of proteins and starch}

Buffer soluble proteins were quantified by using the microassay of Bradford (1976) with BSA
serving as standard. Leaf starch content was determined essentially as described by Abel \textit{et al.}
(1996). Pooled leaf material from several plants was frozen in liquid nitrogen and
homogenized using a mortar. Samples (50 to 80 mg fresh weight of the homogenized frozen
material) were extracted two times with 1 mL 80 [v/v] ethanol for 20 min at 80°C. Insoluble
material was washed with 1 mL water and was then lyophilized. After resuspension in 0.5 mL
200 mM KOH and incubation at 95°C for 1h, the samples were neutralized by adding 1 M
acetic acid and centrifuged (10,000 g for 10 min). Aliquots of the supernatant (50 µL each)
were mixed with 50 µL amyloglucosidase solution (starch determination kit, R-Biopharm,
Darmstadt, Germany) and incubated at 50°C over night. The enzymatic quantification of
glucose was performed following the instructions of the manufacturer.

\textbf{Native PAGE and activity staining}

Native PAGE followed by phosphorylase activity staining was performed as described
elsewhere (Fettke \textit{et al.} 2005a).
Cloning and expression of starch synthase III (AtSSIII; At1g11720)

RNA was isolated from 100 mg *Arabidopsis* leaves, harvested after 3 h illumination, by using the total RNA purification Kit for plant material from Macherey-Nagel (Düren, Germany).

First strand cDNA from *AtsIII* was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) following the manufacturer’s instructions and the 3’ primer (5’-3’:CTTGCGTGCAGAGTGATAGAGCTCAAGATA). Subsequently, the cDNA served as template for the PCR reaction. Both, the EcoRI and XhoI linked primers (5’ forward primer (5’-3‘): GAATCCGATGGAGTGCAGAGTAACGGTCAAGATA and 3’ reverse primer (5’-3‘): CTCGAGCTTGCAGAGTGATAGAGCTGTCGCTGCTCAGAGTAACGGTCAAGATA) include the complete cDNA except the predicted plastidial transit sequence (60 bp from the start). For amplification of the 3.0 kb fragment, a Phusion Taq Polymerase (Finnzymes, Espoo, Finland) was applied.

In a 50 µl reaction volume, 2 µl from the reverse transcription reaction was used as template (30 cycles; annealing temperature 49 °C; 60 sec for extension). Except were stated, the instructions of the manufacturer were followed. The 3.0 kb *AtsIII* fragment was subcloned into pGEM T-easy vector (Promega, Mannheim, Germany). Subsequently, the *AtsIII* fragment was restricted by EcoRI/XhoI and ligated to the expression vector pET23b (Novagen, Darmstadt, Germany). For heterologous expression, the *AtsIII* clone was transformed into *E. coli* BL21 strain. *E. coli* cells were grown at 37 °C in 600 ml culture in Luria-Bertani medium containing 100 µg ml⁻¹ ampicillin until a OD600 = 0.8 was reached.

Expression of the AtSSIII protein was then induced by isopropylthio-β-galactoside (final concentration 0.1 mM; 4 h at 30 °C). Bacterial cells were collected by centrifugation (5,000 x g for 10 min, 4°C), resuspended in 15 ml extraction buffer (20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole, 2.5 mM dithioerythritol [DTE] and protease inhibitor cocktail I; Calbiochem, Bad Soden, Germany), and were then broken by ultrasonification (90 s short pulses). The homogenate was cleared by centrifugation (13, 000 x g for 15 min; 4°C) and the supernatant was loaded on a HisTrap HP column (product no. 17-5319-01; GE Healthcare, Freiburg, Germany). Subsequently, the column was washed with 15 ml extraction buffer and the His-tagged AtSSIII protein was eluted stepwise by increasing concentrations of imidazole (100-500 mM; in extraction buffer pH 8.0). AtSSIII protein containing fractions were identified by western blotting using an anti-His-antibody (Qiagen, Hilden, Germany), pooled and concentrated by ultrafiltration (MWCO 30 kD; Amicon Ultra, Millipore, Schwalbach, Germany). Subsequently, the purified AtSSIII preparation was equilibrated with a buffer containing 50 mM Hepes-NaOH, pH 7.5, 1 mM EDTA and 2 mM
Aliquots of the protein preparation were frozen in liquid nitrogen and were stored at -80°C.

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References


Niewiadomski, P., Knappe, S., Geimer, S., Fischer, K., Schulz, B., Unte, U. S., Rosso, M.
phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac

A previously unknown maltose transporter essential for starch degradation in leaves. *Science*
**303**: 87-9.


Compartmentation of the starch-related R1 protein in higher plants. *Starch* **52**: 179-85.

Cell* **14**: S185-S205.

Satoh H, Shibahara K, Tokunaga T, Nishi A, Tasaki M, Hwang SK, Okita TW, Kaneko
plastidial alpha-glucan phosphorylase gene in rice affects the synthesis and structure of starch

Schneider, A., Häusler, R. E., Kolukisaoglu, U., Kunze, R., van der Graaff, E.,
thaliana knock-out mutant of the chloroplast triose phosphate/phosphate translocator is
severely compromised only when starch synthesis, but not starch mobilisation is abolished.

Schwacke, R., Schneider, A., van der Graaf, E., Fischer, K., Catoni, E., Desimone, M.,


Figure Legends

**Fig. 1** Uptake of external glucose 1-phosphate by *Arabidopsis* mesophyll protoplasts.

(A+B) Short term uptake using varying concentratons of glucose 1-phosphate. Protoplasts were incubated for 30s with [U-¹⁴C]glucose 1-phosphate (concentrations as indicated), washed and the ¹⁴C label inside the protoplasts was determined. The Michealis-Menten (A) and the Lineweaver-Burk (B) plots are given. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given.

(C) Selectivity of glucose 1-phosphate uptake by protoplasts. After 30s incubation of the protoplasts with 200 or 400 µM [U-¹⁴C]glucose 1-phosphate, [U-¹⁴C]glucose 1-phosphate and equal concentration of glucose or glucose 6-phosphate the protoplasts were washed and the total ¹⁴C content of the protoplasts was determined. The mean of three independent experiments and SD are given.

**Fig. 2** Utilization of external glucose 1-phosphate by mesophyll protoplasts from *A. thaliana*.

(A) Glucose or glucose 1-phosphate dependent incorporation into starch. Protoplasts were incubated for 20 min with either [U-¹⁴C]glucose 1-phosphate (G1P; 20 mM) or [U-
14C]glucose (Glc; 20 mM) in the light or in the dark. Subsequently, starch was isolated and the 14C-glucosyl incorporation was monitored. The mean of two independently performed experiments (three replicas each) and the SD are given.

(B) Glucose 1-phosphate uptake and utilization by mesophyll protoplasts. Protoplasts were incubated with [U-14C]glucose 1-phosphate (G1P; 20 mM), [U-14C]glucose 1-phosphate (20 mM) plus unlabelled glucose (10 mM; G1P/Glc), [U-14C]glucose 1-phosphate (20 mM) plus unlabelled glucose 6-phosphate (10 mM; G1P/G6P) or with [U-14C]glucose 1-phosphate (20 mM) plus unlabelled orthophosphate (10 mM; G1P/Pi) for 5 or 10 min in the light. Subsequently, starch was isolated and the 14C-glucosyl incorporation was determined. The mean of three independently performed experiments and SD are given.

(C+D) Counter-exchange of glucose 1-phosphate and orthophosphate. In C protoplasts were incubated for 5 min in the light with [33P]orthophosphate (10 mM; Pi), [33P]orthophosphate (10 mM) and unlabelled glucose 1-phosphate (20 mM; Pi/G1P) or [33P]orthophosphate (10 mM) and unlabelled glucose (20 mM; Pi/Glc). The protoplasts were then washed until no 33P-label could be detected in the washing solution. The 33P content of the protoplasts was monitored. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given. In D protoplasts were incubated for 10 min in the light with [33P]orthophosphate (10 mM). Following removal of the residual external 33P-label by washing, the protoplast suspension was separated into two equal parts. To one part unlabelled glucose 1-phosphate (20 mM) was added (+G1P). In the other part (control) glucose 1-phosphate was omitted (-G1P). After 5 min illumination the protoplasts of both aliquots were pelleted and the 33P content in the supernatant was determined. A typical experiment out of two independently performed experiments (each 3 replicas) and SD is given.

Fig. 3 Glucose 1-phosphate dependent incorporation into starch by chloroplasts from A. thaliana.

(A) Chloroplasts isolated from wild type plants were incubated with [U-14C]glucose 1-phosphate (16.67 mM) in the light. As a control, chloroplasts were broken using a potter and were otherwise treated identically. After incubation the starch was extracted and the 14C content was monitored. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given. intact – intact chloroplasts; broken – mechanically disintegrated chloroplasts were incubated.

(B) Selectivity of the glucose 1-phosphate dependent incorporation into starch. Illuminated chloroplasts were incubated with [U-14C]glucose 1-phosphate (16.67 mM each) or [U-
[U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} plus unlabelled orthophosphate (6.67 mM) or [U-\(^{14}\mathrm{C}\)]\text{glucose 6-phosphate} (16.67 mM each). After isolation of the starch, the \(^{14}\mathrm{C}\)-glucosyl incorporation was determined. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given. G1P – incubation with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate}; G1P/Pi – incubation with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} and unlabelled orthophosphate; G6P – incubation with [U-\(^{14}\mathrm{C}\)]\text{glucose 6-phosphate}.

(C) The glucose 1-phosphate dependent incorporation into starch is unaffected by the addition of unlabelled glucose 6-phosphate or glucose. Illuminated chloroplasts were incubated with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} (16.67 mM each) only (G1P), with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} plus unlabelled glucose 6-phosphate (16.67 mM each; G1P/G6P) or with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} plus unlabelled glucose (16.67 mM each; G1P/Glc). Following 10 or 20 min incubation, starch was isolated and the content of \(^{14}\mathrm{C}\) was quantified. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given.

Fig. 4 Impact of the plastidial phosphorylase isozyme on the incorporation into starch during glucose 1-phosphate incubation.

(A) Phosphorylase pattern as revealed by native PAGE. Leaves were harvested during the light period. Buffer-soluble proteins were extracted from \textit{Arabidopsis} wild type or AtPHS1 knock-out plants. To each lane, 20 µg protein was applied. The separation gel contained 0.4 % [w/v] glycogen. Following electrophoresis, the separation gel was equilibrated, incubated and stained. I – slowly migrating cytosolic phosphorylase (AtPHS2); II – fast migrating cytosolic phosphorylase (AtPHS2); III – slowly migrating plastidial phosphorylase (AtPHS1); IV – fast migrating plastidial phosphorylase (AtPHS1)

(B) Impact of the plastidial phosphorylase (AtPHS1) on the glucose 1-phosphate dependent labelling of starch in \textit{Arabidopsis} mesophyll protoplasts. Protoplasts from wild type (wt) or plastidial phosphorylase knock-out plants (lines Atphs1-1 and Atphs1-2) were incubated with [U-\(^{14}\mathrm{C}\)]\text{glucose 1-phosphate} (20mM). At intervals aliquots of the protoplast suspension were withdrawn and the incorporation of [U-\(^{14}\mathrm{C}\)]glucosyl residues into starch was monitored. The mean of two independently performed experiments (two replicas each) and the SD (n=4) is given.

Fig. 5 Starch synthesis in pgm1 mutants.

(A) Protoplasts were isolated from leaves of wild type plants (wt), a mutant lacking the plastidial phosphoglucomutase activity (pgm1) or having a reduced activity of the
ADPglucose pyrophosphorylase (adg1) and were incubated with [U-^{14}C]glucose 1-phosphate (20 mM) in the light. Subsequently, starch was extracted and the ^{14}C-glucosyl incorporation into the starch was determined. The data represented the mean of three incubations and the SD.

(B+C) Native starch granules isolated from leaves of wild type plants (wt; B) and the pgm1 mutant (C) as analysed by SEM. The bar is equivalent to 20µm.

**Fig. 6 Proposed carbon fluxes in Arabidopsis leaves.**

G1P – glucose 1-phosphate; G6P – glucose 6-phosphate; F6P – fructose 6-phosphate; TP – triose phosphate; ADPG – ADPglucose; SS – starch synthases; AGPase – ADPglucose pyrophosphorylase; pPGM – plastidial phosphoglucomutase

**Tables**

**Table I** ^{14}C incorporation into starch using glucose 1-phosphate in presents of unlabelled hydrogen carbonate. Protoplasts were incubated in presents or absence of 5mM HCO₃⁻ for 30 and 60min, respectively. Protoplasts were illuminated throughout the incubation. The starch was isolated and the ^{14}C incorporation was monitored. Values are given as nmol glc mL⁻¹ (n=3; SD).

<table>
<thead>
<tr>
<th>Incubation time [min]</th>
<th>5 mM HCO₃⁻</th>
<th>no HCO₃⁻ added</th>
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<tr>
<td>30</td>
<td>1.05 ± 0.04</td>
<td>1.72 ± 0.02</td>
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<tr>
<td>60</td>
<td>4.42 ± 0.07</td>
<td>6.17 ± 0.10</td>
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</tbody>
</table>

**Table II** Incorporation of ^{14}C-ADPglucose formed by ADPglucose pyrophosphorylase into starch via starchsynthase III. Protoplasts were incubated with 1mM [U-^{14}C]glucose 1-phosphate in the light or dark. After 30 and 60min incubation the metabolites were extracted and the incorporation of formed ^{14}C-ADPglucose into starch via starchsynthase III was monitored (a). As controls, the starchsynthase III was omitted (minus SSIII) or ^{14}C-labelled ADPglucose were added to the reaction mixture (plus ADPglucose). In addition from the protoplasts the starch was isolated and the ^{14}C incorporation during [U-^{14}C]glucose 1-phosphate incubation was monitored (b). One experiment out of two is shown (n=3, SD).

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>pmol ADPglucose mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>20</td>
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</table>

<table>
<thead>
<tr>
<th>a)</th>
<th>pmol ADPglucose mL⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>10min</td>
<td>20min</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Condition</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>25.79 ± 1.36</td>
<td>28.69 ± 0.67</td>
</tr>
<tr>
<td>dark</td>
<td>1.50 ± 0.12</td>
<td>1.67 ± 0.02</td>
</tr>
<tr>
<td>plus ADPglucose</td>
<td>98039.81 ± 27.56</td>
<td></td>
</tr>
<tr>
<td>minus SSIII</td>
<td>0.48 ± 0.21</td>
<td></td>
</tr>
</tbody>
</table>

b) nmol glc mL$^{-1}$ incorporated into starch:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value 1</th>
<th>Value 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>light</td>
<td>0.097 ± 0.008</td>
<td>0.313 ± 0.011</td>
</tr>
<tr>
<td>dark</td>
<td>0.013 ± 0.009</td>
<td>0.017 ± 0.0012</td>
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</table>
Fig. 1. Uptake of external glucose 1-phosphate by Arabidopsis mesophyll protoplasts.

**(A+B)** Short term uptake using varying concentrations of glucose 1-phosphate. Protoplasts were incubated for 30s with [U-14C]glucose 1-phosphate (concentrations as indicated), washed and the 14C-label inside the protoplasts was determined. The Michealis-Menten (A) and the Lineweaver-Burk (B) plots are given. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given.

**(C)** Specificity of glucose 1-phosphate uptake by protoplasts. After 30s incubation of the protoplasts with 200 or 400 μM [U-14C]glucose 1-phosphate, [U-14C]glucose 1-phosphate and equal concentration of glucose or glucose 6-phosphate the protoplasts were washed and the total 14C-content of the protoplasts was determined. The mean of three independent experiments and SD are given.
Fig. 2. Utilization of external glucose 1-phosphate by Arabidopsis mesophyll protoplasts.

(A) Glucose or glucose 1-phosphate dependent incorporation into starch. Protoplasts were incubated for 20 min with either [U-14C]glucose 1-phosphate (G1P; 20 mM) or [U-14C]glucose (Glc; 20 mM) in the light or in the dark. Subsequently, starch was isolated and the 14C-glucosyl incorporation was monitored. The mean of two independently performed experiments (three replicates each) and the SD are given.

(B) Glucose 1-phosphate uptake and utilization by mesophyll protoplasts. Protoplasts were incubated with [U-14C]glucose 1-phosphate (G1P; 20 mM), [U-14C]glucose 1-phosphate (20 mM) plus unlabeled glucose (10 mM; G1P/Glc), [U-14C]glucose 1-phosphate (20 mM) plus unlabeled glucose 6-phosphate (10 mM; G1P/G6P) or with [U-14C]glucose 1-phosphate (20 mM) plus unlabeled orthophosphate (10 mM; G1P/Pi) for 5 or 10 min in the light. Subsequently, starch was isolated and the 14C-glucosyl incorporation was determined. The mean of three independently performed experiments and SD are given.

(C+D) Counter-exchange of glucose 1-phosphate and orthophosphate. In C protoplasts were incubated for 5 min in the light with 33P-orthophosphate (10 mM; Pi), 33P-orthophosphate (10 mM) and unlabeled glucose 1-phosphate (20 mM; Pi/G1P) or 33P-orthophosphate (10 mM) and unlabeled glucose (20 mM; Pi/Glc). The protoplasts were then washed until no 33P-label could be detected in the washing solution. The 33P-content of the protoplasts was monitored. The mean of two independently performed experiments (two replicates each) and the SD (n=4) are given. In D protoplasts were incubated for 10 min in the light with 33P-orthophosphate (10 mM). Following removal of the residual external 33P-label by washing, the protoplast suspension was separated into two equal parts. To one part unlabeled glucose 1-phosphate (20 mM) was added (+G1P). In the other part (control) glucose 1-phosphate was omitted (-G1P). After 5 min illumination, the protoplasts were pelleted, and the 33P-content in the supernatant was determined. A typical experiment out of two independently performed experiments (each 3 replicates) and SD is given.
Fig. 3 Glucose 1-phosphate dependent incorporation into starch by chloroplasts from *A. thaliana*.

(A) Chloroplasts isolated from wild type plants were incubated with [U-\textsuperscript{14}C]glucose 1-phosphate (16.67 mM) in the light. As a control, chloroplasts were broken using a potter and were otherwise treated identically. After incubation the starch was extracted and the \textsuperscript{14}C-content was monitored. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given. intact - intact chloroplasts; broken - mechanically disintegrated chloroplasts were incubated.

(B) Selectivity of the glucose 1-phosphate dependent incorporation into starch. Illuminated chloroplasts were incubated with [U-\textsuperscript{14}C]glucose 1-phosphate (16.67 mM each) or [U-\textsuperscript{14}C]glucose 1-phosphate plus unlabeled orthophosphate (6.67 mM) or [U-\textsuperscript{14}C]glucose 6-phosphate (16.67 mM each). After isolation of the starch, the \textsuperscript{14}C-glucosyl incorporation was determined. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given. G1P - incubation with [U-\textsuperscript{14}C]glucose 1-phosphate; G1P/Pi - incubation with [U-\textsuperscript{14}C]glucose 1-phosphate and unlabeled orthophosphate; G6P-incubation with [U-\textsuperscript{14}C]glucose 6-phosphate. (C) The glucose 1-phosphate dependent incorporation into starch is unaffected by the addition of unlabeled glucose 6-phosphate or glucose. Illuminated chloroplasts were incubated with [U-\textsuperscript{14}C]glucose 1-phosphate (16.67 mM each) only (G1P), with [U-\textsuperscript{14}C]glucose 1-phosphate plus unlabelled glucose 6-phosphate (16.67 mM each; G1P/G6P) or with [U-\textsuperscript{14}C]glucose 1-phosphate plus unlabelled glucose (16.67 mM each; G1P/Glc). Following 10 or 20 min incubation, starch was isolated and the content of \textsuperscript{14}C was quantified. The mean of two independently performed experiments (two replicas each) and the SD (n=4) are given.
**Fig. 4. Impact of the plastidial phosphorylase isozyme on the incorporation into starch during glucose 1-phosphate incubation.**

**(A)** Phosphorylase pattern as revealed by native PAGE. Leaves were harvested during the light period. Buffer-soluble proteins were extracted from *Arabidopsis* wild type or AtPHS1 knock-out plants. To each lane, 20 µg protein was applied. The separation gel contained 0.4% [w/v] glycogen. Following electrophoresis, the separation gel was equilibrated, incubated and stained as in (A). I – slowly migrating cytosolic phosphorylase (AtPHS2); II – fast migrating cytosolic phosphorylase (AtPHS2); III – slowly migrating plastidial phosphorylase (AtPHS1); IV – fast migrating plastidial phosphorylase (AtPHS1)

**(B)** Impact of the plastidial phosphorylase (AtPHS1) on the glucose 1-phosphate-dependent labelling of starch in *Arabidopsis* mesophyll protoplasts. Protoplasts from wild type (wt) or plastidial phosphorylase knock-out plants (lines Atphs1-1 and Atphs1-2) were incubated with [U-14C]glucose 1-phosphate (20mM). At intervals aliquots of the protoplast suspension were withdrawn and the incorporation of [U-14C]glucosyl residues into starch was monitored. The mean of two independently performed experiments (two replicas each) and the SD (n=4) is given.
Fig. 5 Starch synthesis in pgm1 mutants.

(A) Protoplasts were isolated from leaves of wild type plants (wt), a mutant lacking the plastidial phosphoglucomutase activity (pgm1) or having a reduced activity of the ADPglucose pyrophosphorylase (adg1) and were incubated with [U-^{14}C]glucose 1-phosphate (20 mM) in the light. Subsequently, starch was extracted and the ^{14}C-glucosyl incorporation into the starch was determined. The data represented the mean of three incubations and the SD.

(B+C) Native starch granules isolated from leaves of wild type plants (wt; B) and the pgm1 mutant (C) as analysed by SEM. The bar is equivalent to 20µm.
Fig. 6 Proposed carbon fluxes in Arabidopsis leaves.

G1P – glucose 1-phosphate; G6P – glucose 6-phosphate; F6P – fructose 6-phosphate; TP – triose phosphate; ADPG – ADPglucose; SS – starch synthases; AGPase – ADPglucose pyrophosphorylase; pPGM – plastidial phosphoglucomutase