Glucose-1-Phosphate Transport into Protoplasts and Chloroplasts from Leaves of Arabidopsis

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Almost all glucosyl transfer reactions rely on glucose-1-phosphate (Glc-1-P) that either immediately acts as glucosyl donor or as substrate for the synthesis of the more widely used Glc dinucleotides, ADPglucose or UDPglucose. In this communication, we have analyzed two Glc-1-P-related processes: the carbon flux from externally supplied Glc-1-P to starch by either mesophyll protoplasts or intact chloroplasts from Arabidopsis (Arabidopsis thaliana). When intact protoplasts or chloroplasts are incubated with [U-14C]Glc-1-P, starch is rapidly labeled. Incorporation into starch is unaffected by the addition of unlabeled Glc-6-P or Glc, indicating a selective flux from Glc-1-P to starch. However, illuminated protoplasts incorporate less 14C into starch when unlabeled bicarbonate is supplied in addition to the 14C-labeled Glc-1-P. Mesophyll protoplasts incubated with [U-14C]Glc-1-P incorporate 14C into the plastidial pool of adenosine diphosphoglucose. Protoplasts prepared from leaves of mutants of Arabidopsis that lack either the plastidial phosphorylase or the phosphoglucomutase isozyme incorporate 14C derived from external Glc-1-P 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 extraplastidial Glc-1-P 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 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 cell 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 signaling 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 are 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 Glc 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 (for maltose excess; Niittylä et al., 2004; Weise 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 (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-Suc 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 counterexchange of phosphorylated sugars (or of 3-phosphoglycerate) and orthophosphate. The triose phosphate/phosphate translocator exports triose phosphate (and, to some extent, 3-phosphoglycerate) 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 Glc-6-P/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 Arabidopsis organs (Weber, 2004). Recently, evidence has been presented for the occurrence of an evolutionarily conserved plastidial ADPglucose exporter (Colleoni et al., 2010). However, current knowledge of the metabolite transporters is far from complete, as analysis of the genome of Arabidopsis revealed that approximately 140 putative metabolite transporters exist, all of which are predicted to be located in the inner plastid envelope membrane (Ferro et al., 2002; Schwacke et al., 2003).

None of the translocators described above is able to transport another important metabolite, Glc-1-P (Kammerer et al., 1998; Eicks et al., 2002). This Glc ester is a key intermediate in several major carbon fluxes, such as starch, Suc, and cellulose biosynthesis. In the current model of the photosynthesis-driven starch biosynthesis, the plastidial phosphoglucomutase (pPGM) mediates the formation of Glc-1-P from Glc-6-P, which is directly derived from a Calvin cycle intermediate, Fru-6-P. By the action of ADPglucose pyrophosphorylase, Glc-1-P 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 Arabidopsis lacking the plastidial α-glucan phosphorylase isozyme (PHS1) possess essentially the same starch content as wild-type plants when grown under normal conditions (Zeeman et al., 2004). However, transgenic potato (Solanum tuberosum) plants underexpressing both the plastidial and the cytosolic phosphoglucomutase unexpectedly possess a transitory starch levels similar to those of the wild-type controls. These phenotypic features are considered to be inconsistent with this model (Fernie et al., 2002); therefore, the carbon fluxes toward starch appear to be more complex than are often assumed.

Recently, we have shown that in heterotrophic tissues, such as potato tuber discs, Glc-1-P 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 et al., 2008). Another portion of the imported Glc-1-P 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 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 relies neither on the cytosolic nor the plastidial Glc-6-P/Glc-1-P interconversion.

The question remains whether or not this process operates also in autotrophic tissues, as any evidence for the transport of Glc-1-P into both mesophyll protoplasts and chloroplasts is lacking. In this communication, we have studied the transport of Glc-1-P into autotrophic cells as well as possible implications of this process for the entire transitory starch metabolism.

**RESULTS**

**Uptake and Utilization of External Glc-1-P by Mesophyll Protoplasts from Arabidopsis**

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

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

In order to determine whether or not the imported Glc-1-P is converted to starch (as 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]Glc-1-P or [U-¹⁴C]Glc. During incubation, the protoplasts were either illuminated or darkened. Following incubation, starch was isolated and the ¹⁴C content was quantified. Incubation with Glc-1-P results in a more than 10-fold higher incorporation as compared with Glc (Fig. 2A). Thus, the superior efficiency of the Glc-1-P-dependent labeling of starch is not unique to heterotrophic cells (compare with Fettke et al., 2010). Light stimulates the flux from both external Glc-1-P and Glc to starch, but even in darkened protoplasts, the Glc-1-P-dependent labeling of starch is higher than that of Glc 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 analyzed by ¹⁴C labeling of protoplasts in the light, transfer into the dark, and determination of ¹⁴C label of starch during the dark phase (data not shown). Therefore, in darkened protoplasts, the ¹⁴C labeling of starch equals the total incorporation minus the release of label due to starch degradation.
For a more detailed analysis of the Glc-1-P-dependent incorporation into starch, Arabidopsis mesophyll protoplasts were incubated for 5 or 10 min with one of three mixtures each of which contained [U-14C]Glc-1-P (20 mM each) and, in addition, one of three unlabeled compounds (orthophosphate, Glc, or Glc-6-P; 10 mM each). As a control, an aliquot of the protoplast suspension was incubated with [U-14C]Glc-1-P (20 mM) without adding any unlabeled compound. Protoplasts were illuminated during incubation. The addition of any of the three unlabeled compounds increases the Glc-1-P-dependent incorporation into starch, but quantitatively the enhancement differs depending upon the unlabeled compound. Glc-6-P is more effective than Glc, but orthophosphate is by far most efficient: It increases the labeling of starch approximately 20-fold as compared with the control (Fig. 2B). The stimulatory effect of orthophosphate clearly excludes the possibility that the Glc-1-P-dependent labeling 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 Glc-1-P to starch within 10 min was monitored to be less than 1% as compared with that of the intact protoplasts (data not shown). Furthermore, this type of starch labeling is not expected to be stimulated by light (Fig. 2B; see Fig. 3A below).

During incubation, the Glc-1-P-dependent carbon flux to starch is far from being constant, as most of the 14C 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 labeling (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 Glc-1-P import) by the cytosolic orthophosphate level. If so, the simultaneous addition of orthophosphate and Glc-1-P would lead to a higher cytosolic orthophosphate concentration, which then would stimulate the import of the Glc phosphate.

In order to analyze a possible interdependence of the transport of orthophosphate and of Glc-1-P more directly, two additional labeling 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 [33P]orthophosphate and, in addition, either unlabeled Glc-1-P or unlabeled Glc (20 mM each). As a control, the incubation medium contained only [33P]orthophosphate. Following the incubation for 5 min in the light, the protoplasts were carefully washed to remove external 33P label and the amount of 33P inside the protoplasts was monitored (Fig. 2C). The
addition of unlabeled Glc-1-P decreased the $^{33}$P label inside the protoplasts as compared with the control. By contrast, equimolar external Glc did not affect the $^{33}$P content of the protoplasts. This result is consistent with the assumption that external Glc-1-P is imported into the cell via a Glc-1-P/orthophosphate exchange that takes place at the plasmalemma and thereby diminishes the orthophosphate-dependent prelabeling of the protoplasts.

In the second experiment, protoplasts were prelabeled 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 Glc-1-P. Finally, the protoplasts were pelleted by centrifugation, and the $^{33}$P content in the supernatant was monitored (Fig. 2D). External Glc-1-P increased the release of orthophosphate into the medium more than 2-fold as compared with the control.

In summary, both labeling experiments clearly indicate that the mesophyll protoplasts import external Glc-1-P by an antiport mechanism that utilizes orthophosphate as counter-ion.

**Utilization of External Glc-1-P by Isolated Arabidopsis Chloroplasts**

The results shown in Figure 1 imply that Glc-1-P is taken up by the cells and is converted to starch. This raises the question whether or not Glc-1-P directly enters the chloroplasts where the starch synthesis takes place. Until now, Glc-1-P has not been reported to be imported into chloroplasts, but indirect evidence for an uptake by nongreen plastids has been published (for potato tubers, Kosegarten and Mengel, 1994; Naeem et al., 1997; for wheat [$Triticum aestivum$], Tetlow et al., 1996; for soybean [$Glycine max$], Coates and ap Rees, 1994). To answer the question mentioned above, chloroplasts isolated from Arabidopsis leaves were incubated with $[^{14}C]$Glc-1-P. As a control, an aliquot of the same chloroplast preparation was mechanically broken by using a potter and, subsequently, the homogenate was treated identically. Isolated chloro-

![Figure 2](image-url). Utilization of external Glc-1-P by mesophyll protoplasts from Arabidopsis. A, Glc- or Glc-1-P-dependent incorporation into starch. Protoplasts were incubated for 20 min with either $[^{14}C]$Glc-1-P (G1P; 20 mM) or $[^{14}C]$Glc (Glc; 20 mM) in the light or in the dark. Subsequently, starch was isolated and the $[^{14}C]$glucosyl incorporation was monitored. The mean of two independently performed experiments (three replicas each) and the sd are given. B, Glc-1-P uptake and utilization by mesophyll protoplasts. Protoplasts were incubated with $[^{14}C]$Glc-1-P (G1P; 20 mM), $[^{14}C]$Glc-1-P (20 mM) plus unlabeled Glc (10 mM; G1P/Glc), $[^{14}C]$Glc-1-P (20 mM) plus unlabeled Glc-6-P (10 mM; G1P/G6P), or $[^{14}C]$Glc-1-P (20 mM) plus unlabeled orthophosphate (10 mM; G1P/Pi) for 5 or 10 min in the light. Subsequently, starch was isolated and the $[^{14}C]$glucosyl incorporation was determined. The mean of three independently performed experiments and sd are given. C and D, Counterexchange of Glc-1-P and orthophosphate. C, Protoplasts were incubated for 5 min in the light with $[^{33}P]$orthophosphate (10 mM; Pi), $[^{33}P]$orthophosphate (10 mM) and unlabeled Glc-1-P (20 mM; Pi/G1P), or $[^{33}P]$orthophosphate (10 mM) and unlabeled Glc (20 mM; Pi/Glc). The protoplasts were then washed until no $^{33}$P label could be detected in the washing solution. The $^{33}$P content of the protoplasts was monitored. The mean of two independently performed experiments (two replicas each) and the sd ($n = 4$) are given. D, Protoplasts were incubated for 10 min in the light with $[^{33}P]$orthophosphate (10 mM). Following removal of the residual external $^{33}$P label by washing, the protoplast suspension was separated into two equal parts. To one part, unlabeled Glc-1-P (20 mM) was added (+G1P). In the other part (control), Glc-1-P was omitted (−G1P). After 5 min of illumination, the protoplasts of both aliquots were pelleted and the $^{33}$P content in the supernatant was determined. Results from a typical experiment out of two independently performed experiments (three replicas each) and sd are given.
protoplasts were able to take up Glc-1-P and to convert it into starch at a relatively high rate. This process requires, however, the intactness of the organelles (Fig. 3A). Thus, both the uptake of Glc-1-P and the flux toward starch are functional in isolated intact chloroplasts.

In an additional experiment, chloroplasts were incubated with a mixture of [U-14C]Glc-1-P and (unlabeled) orthophosphate or with [U-14C]Glc-6-P. As a control, chloroplasts were incubated only with [U-14C]Glc-1-P. For the three incubation mixtures, the incorporation of 14C into starch was monitored (Fig. 3B). In the presence of both [U-14C]Glc-1-P and orthophosphate, incorporation into starch is decreased (but still exceeds that observed with Glc-6-P), suggesting a Glc-1-P/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-labeled orthophosphate and unlabeled Glc-1-P results in a decreased uptake of orthophosphate as compared with incubation only with 33P-labeled orthophosphate (data not shown). This result is expected if the external Glc-1-P is imported via an exchange with internal orthophosphate. As the incubation of isolated chloroplast with equimolar [U-14C]Glc-6-P results in a minor labeling of starch (Fig. 3B), import and plastidial metabolism of the two externally supplied Glc phosphate esters differ.

In another series of experiments, isolated chloroplasts were incubated with either [U-14C]Glc-1-P only or together with unlabeled Glc-6-P or Glc (16.67 mM each). 14C incorporation into starch was monitored after 10 or 20 min of incubation. Neither unlabeled Glc-6-P nor free Glc significantly affected the incorporation of 14C into starch, indicating a selective import of the anomeric Glc ester into the chloroplast (Fig. 3C).

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

**Contribution of the Plastidial AtPHS1 to the Conversion of Glc-1-P to Starch**

Following the uptake into chloroplasts, Glc-1-P could be further metabolized by two distinct starch-synthesizing pathways. First, in a single reaction (mediated by the plastidial 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 Glc-1-P 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 isoforms.

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 Glc-1-P (Fig. 4A). On a glycogen-containing separation gel, the two slowly moving bands represent the cytosolic phosphorylase isofrom (AtPHS2) that exists in two states differing in the apparent affinity toward the immobilized polyglucan (Fettke et al., 2005). Similarly, the plastidial phosphorylase isofrom (AtPHS1) occurs in two distinct but faster moving bands (Fig. 4A). In knockout 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 with the wild-type control, mesophyll protoplasts from AtPHS1-deficient lines did not differ in the Glc-1-P-dependent incorporation into starch (Fig. 4B). Therefore, in mesophyll cells, the direct glucosyl transfer to starch, as mediated by AtPHS1, appears to be of no or minor relevance. By contrast, in potato tuber discs, the conversion of Glc-1-P into starch did reflect the level of the plastidial phosphorylase activity (Fettke et al., 2010).

**ADPglucose-Dependent 14C Incorporation into Starch**

As the plastidial Glc-1-P pool is not noticeably used for any AtPHS1-mediated 14C labeling of starch (Fig. 4B), we tested whether or not the conversion of Glc-1-P 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 Glc-1-P toward starch. In order to test this possibility, we incubated protoplasts with 10 mM [U-14C]Glc-1-P in either the presence or absence of 5 mM unlabeled HCO3- (Table I). 14C labeling of starch is significantly reduced by the addition of unlabeled hydrogen carbonate (Table I). These results strongly suggest that the imported Glc-1-P 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-14C]Glc-1-P should result in a 14C labeling of ADPglucose. To test this prediction, we incubated protoplasts derived from wild-type leaves with [U-14C]Glc-1-P 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 of 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 then reacted with a mixture of native potato tuber starch and recombinant starch synthase III (AtSIII) derived from Arabidopsis. Following careful washing, the 14C incorporation into native starch granules was moni-
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 $[^{14}$C]Glc-1-P. In addition, aliquots of the protoplast suspension were used to monitor the incorporation into starch by the intact protoplasts during incubation with $[^{14}$C]Glc-1-P (Table II).

During illumination of protoplasts, import of $[^{14}$C]Glc-1-P results in $^{14}$C incorporation into both starch and ADPglucose. Both processes require intactness of the protoplasts. In the dark, almost no starch is labeled and, in addition, less $^{14}$C-ADPglucose is formed (Table II). 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 Arabidopsis mutant that lacks a functional pPGM (i.e. the *pgm1* mutant) is incapable of performing 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 (Niittylä et al., 2004; Muñoz et al., 2005). This implies that, although to a far lower extent, assimilatory starch can be formed by an additional, yet unknown, path that does not include the plastidial conversion of the Calvin cycle-derived Glc-6-P to Glc-1-P. The import of Glc-1-P into the chloroplast, as analyzed in this study, could be an essential step in this path.

To test this assumption, we prepared mesophyll protoplasts from the *pgm1* mutant and incubated the protoplasts with $[^{14}$C]Glc-1-P during illumination. For comparison, protoplasts were isolated from leaves of two types of Arabidopsis 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).

Figure 3. Glc-1-P-dependent incorporation into starch by chloroplasts from Arabidopsis. A, Chloroplasts isolated from wild-type plants were incubated with $[^{14}$C]Glc-1-P (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 $^{14}$C content was monitored. The mean of two independently performed experiments (two replicas each) and the $\sigma$ ($n$ = 4) are given. Intact indicates intact chloroplasts, and broken indicates mechanically disintegrated chloroplasts were incubated. B, Selectivity of the Glc-1-P-dependent incorporation into starch. Illuminated chloroplasts were incubated with $[^{14}$C]Glc-1-P (16.67 mM each) or $[^{14}$C]Glc-1-P plus unlabeled orthophosphate (6.67 mM) or $[^{14}$C]Glc-6-P (16.67 mM each). After isolation of the starch, the $[^{14}$C]glucosyl incorporation was determined. The mean of two independently performed experiments (two replicas each) and the $\sigma$ ($n$ = 4) are given. G1P, incubation with $[^{14}$C]Glc-1-P; G1P/G6P, incubation with [U-$^{14}$C]Glc-1-P and unlabeled orthophosphate; G6P, incubation with [U-$^{14}$C]Glc-6-P; C, The Glc-1-P-dependent incorporation into starch is unaffected by the addition of unlabeled Glc-6-P or Glc. Illuminated chloroplasts were incubated with $[^{14}$C]Glc-1-P (16.67 mM each) only (G1P), with $[^{14}$C]Glc-1-P plus unlabeled Glc-6-P (16.67 mM each; G1P/G6P), or with $[^{14}$C]Glc-1-P plus unlabeled Glc (16.67 mM each; G1P/Glc). Following 10 or 20 min of incubation, starch was isolated and the content of $^{14}$C was quantified. The mean of two independently performed experiments (two replicas each) and the $\sigma$ ($n$ = 4) are given.
imported into the chloroplast, and then incorporated into starch even in the absence of a functional pPGM. However, labeling of starch was lower as compared with the wild-type control. By contrast, the mutant from Arabidopsis having a lower ADPglucose phosphorylase activity incorporated very little 14C into starch, and labeling was essentially unchanged during the 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 Glc-1-P toward starch.

Regarding the pgm1 mutant, the in vivo flux from Glc-1-P 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 Glc g\(^{-1}\) fresh weight, whereas the ecotype Columbia wild-type plants, grown under the same conditions, contained 6.84 ± 0.427 mg Glc g\(^{-1}\) fresh weight (n = 4). Despite the low starch content of the pgm1 mutant, we were able to isolate native leaf starch granules (Fig. 5C). Based on scanning electron microscopy (SEM) examination, the morphology of the mutant-derived starch particles is similar to that obtained from wild-type leaves (Fig. 5B; Streb et al., 2009).

For several reasons, the relatively small contribution of the Glc-6-P-independent flux to the total starch biosynthesis is not surprising: First, in the cytosol, Glc-1-P 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 Suc and cell wall polysaccharides. Second, the total cellular content of Glc-1-P is very low and undergoes only moderate changes throughout the light/dark cycle (Schneider et al., 2002). Finally, provided an appropriate gradient is given, an efficient Glc-1-P/phosphate antiporter located in the chloroplast envelope membrane(s) will even lower the plastidial Glc-1-P pool if mediating 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 Arabidopsis are capable of utilizing extracellular Glc-1-P. Following uptake, Glc-1-P passes the plastidial envelope membranes and, finally, the hexosyl residue is converted to starch.

**Table 1.** 14C incorporation into starch using Glc-1-P in the presence of unlabeled hydrogen carbonate

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<th>Incubation Time</th>
<th>5 mM HCO(_3^-)</th>
<th>No HCO(_3^-) Added</th>
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<tr>
<td>30 min</td>
<td>1.05 ± 0.04</td>
<td>1.72 ± 0.02</td>
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<tr>
<td>60 min</td>
<td>4.42 ± 0.07</td>
<td>6.17 ± 0.10</td>
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Protoplasts were incubated in the presence or absence of 5 mM HCO\(_3^-\) for 30 and 60 min, respectively. Protoplasts were illuminated throughout the incubation. The starch was isolated, and 14C incorporation was monitored. Values are given as nmol Glc mL\(^{-1}\) (n = 3; ±SD).
Table II. Incorporation of \([^{14}C]\)ADPglucose formed by ADPglucose pyrophosphorylase into starch via AtSSIII

<table>
<thead>
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<th>Treatment</th>
<th>10 min</th>
<th>20 min</th>
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<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>
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<tr>
<td>Plus ADPglucose</td>
<td>98,039.81 ± 27.56</td>
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<tr>
<td>Minus SSIII</td>
<td>0.48 ± 0.21</td>
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The transfer of glucosyl residues to nonreducing 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 following equation: ATP + Glc-1-P ⇌ ADPglucose + PP₃.

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

To some extent, the data reported here concur with the metabolism of Glc-1-P as described for heterotrophic tissues, such as potato tubers (Fettke et al., 2008, 2010). Tuber parenchyma cells are capable of importing Glc-1-P and incorporating 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.

![Figure 5](https://www.plantphysiol.org)
of the cytosolic phosphorylase did not affect the \([U-14C]\) carbon flux directed to starch, as a reduced activity of this reaction seems to be of minor relevance for the incorporated, via the cytosolic phosphorylase isozyme, into membranes, respectively (Fettke et al., 2010). In addition, the imported Glc-1-P 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 \([U-14C]\) Glc-1-P-dependent labeling of starch. Nevertheless, this pathway could be involved in buffering the cytosolic Glc-1-P pool.

Following the import of Glc-1-P 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, Glc-1-P imported into the chloroplast leads to the formation of ADPglucose, which then acts as a glucosyl donor for starch synthases (Table II). These results are in agreement with the conclusion that in Arabidopsis, 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 plants having a reduced pPGM activity accumulate by far less reserve starch as compared with wild-type controls (Tauberger et al., 2000).

Similarly, Arabidopsis plants (and potato leaves as well) lacking a functional pPGM (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; Streb et al., 2009). However, potato lines possessing an antisense repression of both the cytosolic PGM and the pPGM unexpectedly exhibit a phenotype similar to the wild type (Fernie et al., 2002). Similarly, the \(sta5-1\) mutant from \textit{Chlamydomonas reinhardtii}, which is reported to lack the pPGM, accumulates 4% to 12% of the normal starch amounts (Van den Koornhuyse et al., 1996). In all these cases, an import of Glc-1-P into the plastid can, at least partially, compensate the blocked Glc-6-P/Glc-1-P 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. However, the quantification of the two Glc esters in several tissues indicates that the Glc-6-P levels exceed those of Glc-1-P (Tarnowsky et al., 1964; Alpers, 1968; Tetlow et al., 1998). Thus, the import of Glc-1-P into the plastids appears to be limited by the cytosolic concentration of the substrate. Interestingly, in mutants lacking a functional phosphoglucomutase, the Glc-6-P content is increased 10-fold but that of Glc-1-P is only slightly higher as compared with 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 central carbon metabolism. In pPGM-lacking mutants of Arabidopsis, the plastidial transport activity for both Glc-6-P and phosphoglycerate is significantly increased (Kunz et al., 2010). Possibly, the flux from the plastidial Glc-6-P pool into the cytosol is enhanced, which results in a faster formation of Glc-1-P by the cytosolic phosphoglucomutase 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 the Glc-6-P transporter is weak (Niewiadomski et al., 2005). However, for the plastidial Glc-6-P/phosphate translocator mutants, both a strongly increased expression of a second isoform of this transporter and an enhanced transport rate have been reported (Kunz et al., 2010).

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

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

The Glc-1-P-dependent starch labeling in mesophyll protoplasts is much higher than that observed during incubation with Glc (Fig. 2). Surprisingly, simultaneous incubation of the protoplasts with \([U-14C]\)Glc-1-P and orthophosphate results in a strongly increased

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**Figure 6.** Proposed carbon fluxes in Arabidopsis leaves. ADPG, ADPglucose; AGPase, ADPglucose pyrophosphorylase; F6P, Fru-6-P; G1P, Glc-1-P; G6P, Glc-6-P; pPGM, plastidial phosphoglucomutase; SS, starch synthases; TP, triose phosphate.
labeling of starch. This enhancement suggests an efficient limitation of Glc-1-P import by the cytosolic orthophosphate level. However, the simultaneous incubation with [U-13C]Glc-1-P and Glc or Glc-6-P also results in an enhanced labeling of starch. Currently, this effect is difficult to explain. However, it should be taken into consideration that metabolic paths are often superimposed by sugar-mediated signaling effects that alter carbon fluxes (see above).

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

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) plants (ecotype Columbia, Wassilewskija, pgm1, Atphs1-1, Atphs1-2) were grown in growth chambers using either a 12-h-light/12-h-dark (16°C) or a 14-h-light (22°C)/12-h-dark (16°C) cycle. Throughout the light/dark cycles, relative humidity was 60%.

Protoplast Preparation

Mesophyll protoplasts were prepared from Arabidopsis plants grown for 3 to 4 weeks in leaves. Leaves (3–4 g each) that had been washed with water were transferred into 400 mM mannitol dissolved in water. Following slicing (approximately 4 mm thickness), the leaf material was incubated for 3 to 3.5 h at 25°C under continuous gentle shaking in an incubation medium consisting of 5 mM MES-KOH, pH 5.6, 400 mM mannitol, 8 mM CaCl2, 1% (v/v) ethanol and decolorized at 70°C. The samples were centrifuged as above, and the pellets were collected. Following the addition of 1 mL of water each, the suspensions were mixed and centrifuged as above. Subsequently, 500 μL of 200 mM KOH was added to each pellet, and the mixtures were incubated for 1 h at 95°C. Following neutralization with 1 mM acetic acid, the samples were centrifuged as above, and the 14C content of the supernatants was monitored by using a liquid scintillation counter (Beckman Coulter). As revealed by treatment with amyloglucosidase, more than 98% of the 14C-labeled material solubilized by KOH consists of α-polygalactans; therefore, this fraction is designated as starch.

Exchange Experiments

The orthophosphate-related exchange was analyzed by two types of experiments. Throughout both types of experiments, the protoplasts were illuminated (80 μmol m–2 s–1, room temperature). For type 1, resuspended protoplasts (each 15 mL) were incubated for 5 min in the presence of 10 mM ([U-14C]ADPglucose) or in a mixture containing 10 mM (185 kBq) [33P]orthophosphate and 20 mM unlabeled Glc-1-P. The protoplasts were then repeatedly washed with resuspension medium until the total radioactivity in the washing solution was below 100 dpm. The pelletted protoplasts were then resuspended in 1 mL of water, and the radioactivity was monitored using a liquid scintillation counter. For type 2, protoplasts (30 mL) were incubated for 10 min with 1.85 MBq [33P]orthophosphate (specific activity of 3,000 Ci mmol–1) and, subsequently, were washed three times with resuspension medium. The protoplast suspension was then divided into two equal parts. One part was incubated for 5 min in the presence of 20 mM unlabeled Glc-1-P. The other part (control) was incubated for 5 min in the absence of Glc-1-P. Following incubation, the protoplasts were pelleted by centrifugation (90g for 12 min), and in the supernatant, the [33P] content was determined using a liquid scintillation counter.

Short-Term Uptake Experiments

For short-time uptake experiments, protoplasts (4 mL) were incubated with Glc-1-P, Glc-1-P and Glc, or Glc-1-P and Glc-6-P (concentration as indicated, and 37 kBq [U-13C]Glc-1-P was added) for 30 s at room temperature and illumination. The protoplasts were then immediately centrifuged (90g for 3 min at 4°C), and the supernatant was discarded. The pelletted protoplasts were resuspended in 8 mL of resuspension medium and centrifuged again (90g for 3 min at 4°C). This washing step was repeated two times. Finally, 1 mL of water was added to the pelletted protoplasts, and the total 14C content was quantified by liquid scintillation counting.

Quantification of [14C]ADPglucose

Protoplasts were incubated in a resuspension medium containing 1 μM unlabeled Glc-1-P and 74 kBq [U-13C]Glc-1-P in the light or in the dark. In the latter case, protoplasts were predarkened for 1 h. After 10 or 20 min of incubation, the entire suspension was immediately frozen in liquid nitrogen. Following the addition of ethanol (final concentration of 80% [v/v]), the suspension was heated (10 min at 90°C). Following cooling and centrifugation (10,000g for 10 min), each pellet was used for isolation of starch and monitoring of the 13C content (see above). Each supernatant (containing soluble metabolites plus externally supplied compounds) was lyophilized and then dissolved in 3.5 mL of water. Then, 1.5 mL of each solution was added to the reaction buffer (as final concentration, 40 mM Tricine, pH 8.0, 1.6 mM EDTA, 20 mM potassium acetate, 75 μM citrate, and 13 mg of native potato (Solana tuberosum) tuber starch). The glucosyl transfer from ADPglucose to the native starch granules was started by the addition of 17 μg of recombinant AtSSIII and was continued for 30 min at 30°C. Subsequently, 25 μmol of unlabeled ADPglucose and 5 μg of AtSSIII were added, and the mixture was incubated for an additional 1 h. As controls, either the AtSSIII was omitted (negative control) or, alternatively, 8.3 kBq [U-13C]ADPglucose (positive
control was added during incubation. Finally, the samples were centrifuged (14,000g for 2 min), and the pelletted starch was washed six times by resuspending in 1 mL of water each and centrifugation (as above). In the pelletted starch, the total 14C content was quantified by liquid scintillation counting.

Chloroplast Isolation and Labeling

Chloroplasts were prepared according to Shi et al. (2000) with minor modifications. Approximately 2 g of Arabidopsis leaves was harvested in the beginning of the light period, freed of midveins, cut into small slices, and incubated with 50 mL of precooled isolation buffer containing 20 mM Tricine-NaOH, pH 8.4, 300 mM sorbitol, 10 mM EDTA, 10 mM KCl, 0.25% (v/v) bovine serum albumin, 5 mM sodium ascorbate, and 5 mM dithioerythritol (DTE). The slices were homogenized two times for 3 s each using a Waring Blender, and the resulting homogenate was filtered through two layers of Miracloth (Calbiochem-Novabiochem). The filtrate was centrifuged (750g for 1 min at 4°C), and the pelletted chloroplasts were resuspending in precooled isolation buffer (as above). The isolated chloroplasts were incubated with [U-14C]Glc-1-P (16.67 mM, containing 74 kBq; final volume of 4 mL) plus unlabeled 6.67 mM orthophosphate or Glc-6-P (16.67 mM, containing 74 kBq; final volume of 4 mL) or [U-14C]Glucose (16.67 mM) or [U-14C]Glc-1-P (16.67 mM, containing 74 kBq; final volume of 4 mL) or [U-14C]Glc (16.67 mM) or [U-14C]Glc-1-P (16.67 mM, containing 74 kBq; final volume of 4 mL) plus unlabeled Glc (16.67 mM) or [U-14C]Glc-1-P (16.67 mM, containing 74 kBq; final volume of 4 mL) plus unlabeled Glc-6-P (16.67 mM). Throughout the experiments, the chloroplasts were incubated at 80°C for 1 min at 4°C. Following centrifugation (as above), the pellets were resuspended in 20% (v/v) ethanol and centrifuged again as above. The pellets were decolorized in 80% (v/v) ethanol at 70°C (20 min). After centrifugation (as above), the pellets were washed three times with 80% (v/v) ethanol. The resulting pellets were treated with 200 mM KOH for 1 h at 95°C. Following neutralization 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 pswII mutant plants was harvested and immediately frozen in liquid nitrogen. Following homogenization using a mortar, native starch was isolated according to Ritte et al. (2000) with minor modifications. Following a passage through a Percoll cushion (4,000 g for 15 min at 4°C), the pelletted starch was washed twice with extraction buffer (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 mixture (1:1, v/v) to the starchy suspension and centrifugation (9,000 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 13,000g, and the pelletted starch was collected.

Potato tuber starch was isolated according to Ritte et al. (2000). Potato tuber tissue (15–20 g) was mixed with 50 mL of 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 a 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 five 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).

Extraction of Buffer-Soluble Proteins

Leaf material was frozen in liquid nitrogen and homogenized using a mortar. Per 1 g fresh weight, 1 mL of precooled buffer A (100 mM HEPES-NaOH, pH 7.5, 1 mM EDTA, 2 mM DTE, 30% [v/v] glycerol, 0.5 M phenylmethylsulfonyl fluoride) was added. All subsequent steps were performed at 4°C. The resulting homogenates were centrifuged (20,000g 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.

Quantification of Proteins and Starch

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

Native PAGE and Activity Staining

Native PAGE followed by phosphorylase activity staining was performed as described elsewhere (Fettke et al., 2005).

Cloning and Expression of AtSSIII

RNA was isolated from 100 mg of Arabidopsis leaves, harvested after 3 h of illumination, by using the total RNA purification kit for plant material from Macherey-Nagel. First-strand cDNA from AtssIII was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer’s instructions and the 3′ primer (5′-CTTGGTCTAGGTAAGCTAGATA-GATA-3′). Subsequently, the cDNA served as a template for PCR. Both the EcoRI- and XhoI-linked primers (5′forward primer, 5′-GAATCCGATGGAAGT- CTCAGAAAGAACAAC-3′, and 3′ reverse primer, 5′-CTCGAGCTTACTCCGTA-GAGTCGATAGCTTCC-3′) 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) was applied. In a 50-μL reaction volume, 2 μL from the reverse transcription reaction was used as a template (30 cycles, annealing temperature of 49°C, 60 s for extension). Except where stated, the instructions of the manufacturer were followed. The 3.0-kb AtssIII fragment was subcloned into pGEM T-Easy vector (Promega). Subsequently, the AtssIII fragment was restricted by EcoRI/XhoI and ligated to the expression vector PET23b (Novagen). For heterologous expression, the AtssIII clone was transformed into Escherichia coli strain BL21. E. coli cells were grown at 37°C in 600 mL of culture in Luria-Bertani medium containing 100 μg mL−1 ampicillin until an optical density at 600 nm = 0.9 was reached. Expression of the AtssIII protein (AtssIII) was then induced by isopropyl-β-D-thiogalactoside (final concentration of 0.1 mM 4 h at 30°C). Bacterial cells were collected by centrifugation (5,000g for 10 min at 4°C), resuspended in 15 mL of extraction buffer (20 mM sodium phosphate buffer, pH 8.0, 500 mM NaCl, 20 mM imidazole, 2.5 mM DTE, and protease inhibitor cocktail I; Calbiochem), and then broken by ultrasonication (90-s short pulses). The homogenate was cleared by centrifugation (13,000g for 15 min at 4°C), and the supernatant was loaded on a HiTrap HP column (product no. 17-5319-01; GE Healthcare). Subsequently, the column was washed with 15 mL of 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 (Quantigen), pooled, and concentrated by ultrafiltration (30 kD; Amicon Ultra; Millipore). Subsequently, the purified AtSSIII preparation was equilibrated with a buffer containing 50 mM NaOH, pH 7.5, 1 mM EDTA, and 2 mM DTE. Aliquots of the protein preparation were frozen in liquid nitrogen and stored at −80°C.

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