A Novel Type of Chloroplast Stromal Hexokinase Is the Major Glucose-phosphorylating Enzyme in the Moss Physcomitrella patens*

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Hexokinase catalyzes the first step in the metabolism of glucose but has also been proposed to be involved in sugar sensing and signaling both in yeast and in plants. We have cloned a hexokinase gene, PpHXK1, in the moss Physcomitrella patens where gene function can be studied directly by gene targeting. PpHxk1 is a novel type of chloroplast stromal hexokinase that differs from previously studied membrane-bound plant hexokinases. Enzyme assays on a knock-out mutant revealed that PpHxk1 is the major glucose-phosphorylating enzyme in Physcomitrella, accounting for 80% of the total activity in protonemal tissue. The mutant is deficient in the response to glucose, which in wild type moss induces the formation of caulonemal filaments that protrude from the edge of the colony. Growth on glucose in the dark is strongly reduced in the mutant. Sequence data suggest that most plants including Physcomitrella and Arabidopsis have both chloroplast-imported hexokinases similar to PpHxk1 and traditional membrane-bound hexokinases. We propose that the two types of plant hexokinases have distinct physiological roles.

Hexokinase catalyzes the first step in the glycolysis, the phosphorylation of glucose or fructose. Biochemical experiments suggest that there are several hexokinases in plants that differ in their intracellular locations. Thus, in addition to a cytosolic activity, hexokinases have been reported to be associated with mitochondria (1–3), the Golgi complex (3), and chloroplasts (2, 4–6). Plant hexokinases that have been cloned and studied in some detail include Arabidopsis AtHxk1 and AtHxk2 (7), spinach SoHxK1 (2), and potato StHK1 and StHK2 (8, 9). All of these plant hexokinases share an N-terminal hydrophobic membrane anchor, which is not present in yeast and animal hexokinases. The intracellular location has been studied for SoHxK1, which is inserted into the outer envelope membrane of the chloroplast, facing the cytoplasmic side (2). This insertion is mediated by the N-terminal membrane anchor that is conserved in AtHxk1, AtHxk2, StHK1, and StHK2 (2, 9). Therefore, it is likely that these other plant hexokinases also are membrane-bound, similar to SoHxK1.

In addition to its enzymatic role, hexokinase has been implicated in sugar sensing and signaling in several organisms (10, 11). This notion originated with the finding that hexokinase is essential for glucose repression (12), a key regulatory mechanism in yeast (13, 14). However, attempts to identify the glucose-dependent signal that is transmitted by hexokinase have so far failed and its role in glucose repression is still not fully understood (15). In plants, sugars act as regulatory signals at several points in the life cycle (16) and can also repress a number of genes involved in, for example, photosynthesis (17). Arabidopsis plants that overexpress hexokinase are sugar hypersensitive, whereas plants expressing hexokinase antisense RNA show hyposensitivity to sugars (7). Therefore, it has been proposed that plant hexokinases play a role in sugar sensing or signaling that is analogous to the role of yeast hexokinase in glucose repression (7). However, this notion was challenged by Halford et al. (18) who pointed out that many sugar-responsive processes are affected by changes in the ATP level. Some effects cited as evidence of a role for hexokinase in sugar sensing could therefore just reflect its function as a rate-limiting enzyme in glucose metabolism. The question regarding the role of plant hexokinases in sugar sensing is complex (19, 20), but recent experiments with AtHxk1 point mutations support distinct metabolic and signaling functions for this enzyme (21).

The moss Physcomitrella patens has recently emerged as a powerful model system in plant functional genomics following the discovery that gene targeting works in it with frequencies comparable with those in yeast (22, 23). Furthermore, the main vegetative phase of Physcomitrella is the haploid gametophyte, which makes it possible to study the phenotypes of knock-outs directly without further crosses. To study the function of hexokinases in plants, we have cloned a full-length cDNA and gene, PpHXK1, encoding a hexokinase in Physcomitrella. PpHxk1 is a novel type of chloroplast stromal hexokinase, which differs from the membrane-associated enzymes represented by SoHxK1. Enzyme assays on a knock-out mutant revealed that PpHxk1 is the major glucose-phosphorylating enzyme in Physcomitrella, accounting for 80% of the total activity in extracts. The hxxk1 knock-out mutant is deficient in a response to intermediate levels of glucose, which involves the induction of caulonemal filaments that protrude from the colony. Growth on glucose in the dark is also severely impaired in the mutant. Sequence data suggest that both chloroplast stromal hexokinases similar to PpHxk1 and traditional membrane-bound hexokinases are present in most plants.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—The standard growth conditions used in this work were grown at 25 °C under constant light in...
Novel Chloroplast Hexokinase in Physcomitrella

TABLE I

| Name          | Oligonucleotides                  |
|---------------|-----------------------------------|
| HXKdeg-5'     | gtagttatggcagttgtagagtgaagcgattgg |
| HXKdeg-3'     | cacggcttggaatctggagaacggcggagc   |
| PpHXK1-5'A    | cacgtgctgacggtgcaggtgcaggtgcaggtg |
| PpHXK1-3'B    | caatggaattctgatgagatggacnaarggntt |
| PpHXK1-3'B    | cacgtgctgacggtgcaggtgcaggtgcaggtg |
| PpHXK1-RT-5'  | ggaagttgctttggaaatcggtagaagct    |
| PpHXK1-R-3'   | gacacgagcgaagggaaggggttcacagtc   |
| PpHXK1/GFP-5'A| ccagcttgctgacggtgcaggtgcaggtgcaggtg |
| PpHXK1/GFP-5'B| gtagttatggcagttgtagagtgaagcgattgg |
| PpHXK1/GFP-3'C| ggcggcatactgcaaaccaaatct    |

The abbreviations used are: RACE, rapid amplification of cDNA ends; GFP, green fluorescent protein; RT, reverse transcriptase; Glc-6-P, glucose 6-phosphate; TRITC, tetramethylrhodamine isothiocyanate.

Southern and Northern Blot Analysis—P. patens genomic DNA was isolated from young protonemal tissue and digested to completion with HindIII, BglII, or EcoRI. Using standard blotting procedures, the DNA was transferred to a nylon filter that was UV cross-linked. The filter was hybridized in rapid-hyb buffer (RPN1636, Amersham Biosciences) to a 32P-labeled probe for 4 h at 65 °C and then washed with 0.5 × SSC + 0.1% SDS at 65 °C. A 460-bp PstI-SphI fragment from the PpHXK1 cDNA was used as the probe. Hybridizing bands were visualized using a PhosphorImager. Northern blot analysis was carried out with total RNA from young protonemal tissue grown on BCD medium under standard conditions. Approximately, 30 μg of total RNA was loaded in each lane. The probe template was a 566-bp PcoI-BglII fragment from the PpHXK1 cDNA. Ethidium bromide staining of rRNA was used to check for equal loading of RNA in each lane.

Isolation and Fractionation of Chloroplasts—Protoplasts were produced by the digestion of 2–4 g of young protonemal tissue for 30 min in 1% driselase (Sigma, D95215), 8% mannitol. The protoplasts were then separated from undigested material by filtration through a 100-μm nylon mesh, and after several washes in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM EDTA, and 0.3 mM mannitol), they were disrupted by forcing them through a 15-μm nylon mesh. The disrupted protoplasts were loaded onto a Percoll step gradient in buffer A (10:30:60% v/v). After centrifugation in a swing-out rotor for 10 min at 44500 × g, the major chloroplast band was collected at the interface between 60 and 30% Percoll. The chloroplasts were washed in buffer A to remove the Percoll and were then redissolved in 4 ml of buffer A. Phase-contrast light microscopy was used to confirm that the collected fraction consisted of chloroplasts and that most of them were still intact.

Half of the chloroplasts were pelleted by centrifugation and then solubilized in 2 ml of buffer AT (50 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 1 mM EDTA, and 1% Triton X-100). Debris was removed by centrifugation for 10 min at 13,000 rpm to produce a chloroplast extract. The total protein content in the extract was determined using the Bradford assay (Bio-Rad 500-0006). The remaining half of the chloroplasts were pelleted by centrifugation and then solubilized in 2 ml of buffer AT after the debris was removed by centrifugation for 10 min at 13,000 rpm, thus producing the chloroplast membrane fraction. Extracts were assayed for glucose-phosphorylating activity as soon as possible after preparation since we found that the activity in chloroplast membrane-containing fractions declined rapidly with time.

Hexokinase Assays—The protocol was adopted from Wiese et al. (2) with some modifications. To prepare crude cellular extracts, young protonemal tissue (400 mg) grown on celllophane-covered BCD plates was ground to a fine powder in liquid nitrogen and then further homogenized in 2 ml of AT buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM EDTA, and 1% Triton X-100). Cell debris was removed by centrifugation for 10 min at 13,000 rpm. The total protein content of the crude extract was determined using the Bradford assay (Bio-Rad 500–0006). To assay glucose-phosphorylating activity, extracts (crude, chloroplast, chloroplast stroma, or chloroplast membranes) were diluted to a total volume of 100 μl in 100 mM HEPES-KOH, pH 7.8, 10 mM MgCl2, 2 mM ATP, 0.8 mM NADP, 0.2 units of glucose-6-phosphate dehydrogenase (Sigma G5760), and 10 mM glucose. To assay fructose-phosphorylating activity, 0.2 units of fructose-6-phosphate isomerase (128139, Roche Applied Science) was added and the substrate was changed from glucose to 10 mM fructose. Furthermore, a different preparation of glucose-6-phosphate dehydrogenase (Sigma G8529) was used to minimize the extract-independent background. The samples were incubated at 30 °C for 20

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min unless stated otherwise, after which the NADPH formed was measured at 340 nm using an extinction coefficient of 6200 M⁻¹ cm⁻¹. The NADPH values were in turn converted into moles of glucose-6-phosphate formed.

**Phenotypic Analysis**—For colony morphology studies, protonemal tissue was precultured for 1 week on cellophane-covered ammonium-free BCD medium. Small pieces of fresh protonemal tissue (~2 mm in diameter) were then transferred onto ammonium-free BCD plates without cellophane, containing various additives as described in the figures. The colonies were cultivated in constant light for 21 days and then photographed using a dissecting microscope. To analyze the morphology of dark grown colonies, small pieces of fresh protonemal tissue (~2 mm in diameter) were inoculated on BCD plates supplemented with 0.4% or 0.15% glucose. The colonies were grown under standard conditions for 7 days. The plates were then placed vertically and cultivated in darkness for additionally 30 days before being photographed using a dissecting microscope.

**Quantitative RT-PCR Analysis of mRNA levels**—Young protonemal tissue cultivated for 7 days on cellophane-covered BCD plates under standard growth conditions was transferred by disc lifts to tester plates. The conditions tested included growth in the dark or under intense light (6000 lux) and growth in the presence of 0.2 µM glucose or 0.2 µM mannanitol. Each treatment was done in triplicate. After 24 h of treatment, the tissue was harvested and total RNA was isolated. Expression levels were measured as the ratio between the transcript-dependent RT-PCR product in 1 µg of total RNA and an internal standard product. The internal standard is added as *in vitro* transcribed RNA that is amplified with the same primer pair as the transcript of interest but produces a differently sized product (27). The primers used were PpHXK1-RT-5’ and PpHXK1-RT-3’ (Table I).

**RESULTS**

**Cloning and Characterization of the PpHXK1 cDNA and Gene**—Based on published hexokinase sequences from plants, we designed degenerate primers that were used in a PCR with *P. patens* genomic DNA as the template. Using this approach, we cloned several short fragments, one of which was derived from a gene that we named *PpHXK1* and which shows clear homology to other hexokinases. *PpHXK1*-specific primers were then used with cDNA from moss grown under standard conditions in 5’ and 3’ RACE reactions. Based on the sequences of the resulting products, we could design the primers 5’A and 3’A (Table I), which were used to amplify a full-length cDNA clone and the corresponding gene. Several *Physcomitrella* genes have been found to have a gene organization that is identical to their higher plant homologs (23). Consistent with this finding, we found that the exon/intron distribution of *PpHXK1* (Fig. 1A) is identical to that of the *Arabidopsis* *AtHXK2* gene (7).

To determine whether there are any other genes in the *Physcomitrella* genome that are closely related to *PpHXK1*, we performed a low stringency Southern blot with a cDNA probe recognizing the 3’ half of the *PpHXK1*-coding sequence. We found a single strongly hybridizing band in each lane that matched the size expected for the *PpHXK1* gene (data not shown). In some lanes, there was also an additional faint band, indicating the presence of another more distantly related hexokinase gene. This finding is consistent with our recovery of other hexokinase-encoding sequences in the initial PCR reaction. An evolutionary tree based on pairwise amino acid sequence comparisons (Fig. 1B) places PpHxk1 among the other plant hexokinases. It is 85% similar and 53% identical in sequence to the *Arabidopsis* AtHxk1 and AtHxk2 proteins, whereas a comparison to the yeast Hxk1 and Hxk2 protein yielded 72% similar and 34% identical residues.

**PpHxk1 Is the Major Glucose-phosphorylating Enzyme in Young Protonemal Tissue**—To investigate the function of *PpHXK1*, we made a disruption construct (Fig. 2A) that was used to knock out the gene (26). The presence of the desired gene disruptions in moss chromosomal DNA was verified by PCR (Fig. 2B), and the disappearance of the *PpHXK1* transcript was confirmed in Northern blots (Fig. 2C). Interestingly, the PCR analysis revealed that one of the knock-outs was a clean single copy insertion of the disruption marker. This is unusual in *Physcomitrella* where most knock-out mutants carry multiple tandem repeats of the inserted marker (23). An initial phenotypic characterization was carried out with three independent knock-outs. Since these three mutants had the same phenotypes, further experiments were restricted to the single copy insertion mutant, which is referred to as the *hxk1* mutant below.

We proceeded to test whether we could detect a difference in glucose-phosphorylating activity between extracts of young protonemal tissue derived from the *hxk1* mutant and the wild type. Using standard growth conditions, we found that wild type extracts have a glucose-phosphorylating activity of 40 pmol/min and µg of total protein. This activity is linear in the amount of added protein below 5 µg (Fig. 3A) and also linear in time (Fig. 3B). Interestingly, we further found that most of this activity is absent in extracts from the *hxk1* mutant (Fig. 3B). However, it should be noted that some glucose-phosphorylating activity remains in the *hxk1* mutant and that this activity also is linear in the amount of added protein (Fig. 3A) and time (Fig. 3B). This indicates the presence of one or several minor hexokinases in addition to PpHxk1. The residual glucose-phospho-
rlyating activity in the hxl mutant is 8.6 pmol/min and μg of protein, which accounts for 22% of the wild type activity. We conclude that PpHxk1 is the major glucose-phosphorylating enzyme in young protonemal tissue, accounting for 78% of the total glucokinase activity in these cells.

Many hexokinases have a dual specificity for glucose and fructose. Therefore, we proceeded to assay fructokinase activity in protonemal extracts from the wild type and the hxl mutant (Fig. 3, C and D). As shown in Fig. 3D, we found that the fructokinase activity is 24.7 pmol/min and μg of total protein in the wild type and 13.0 pmol/min and μg of total protein in the hxl mutant. We conclude that PpHxk1 can phosphorylate both glucose and fructose and that it contributes 47% of the total fructokinase activity in protonemal tissue. The comparatively smaller effect of the hxl mutation on fructose phosphorylation does not necessarily mean that fructose is a less preferred substrate for PpHxk1 than glucose, because plants in addition to classical hexokinases like those in animals and fungi also possess specific fructokinases, which are more distantly related to the hexokinases. Therefore, it is probable that the effect of the hxl mutation on fructose phosphorylation in part is obscured by the presence of such specific fructokinases in the extracts.

PpHxk1 Is a Chloroplast Stromal Enzyme Whose Import Depends on an N-terminal Transit Peptide—The presence of an N-terminal extension in PpHxk1, which is absent from both Hxk1 and AtHxk2, prompted us to analyze the PpHxk1 sequence using the TargetP software for prediction of subcellular localization (29). The result suggested that PpHxk1 could be a chloroplast-imported protein with a possible transit peptide cleavage site after amino acid residue 37 (30). To test this prediction, we made plasmids in which different PpHxk1 fragments expressed from the 3SS promoter were fused in-frame to the GFP. These constructs were transiently transformed into wild type Physcomitrella protoplasts after which the pattern of GFP fluorescence was analyzed. Chlorophyll autofluorescence was used as a chloroplast marker. As shown in Fig. 4, GFP alone resides in the cytoplasm and is clearly excluded from the chloroplasts. In contrast, when GFP is fused to an almost full-length PpHxk1 (amino acid residues 1–37), shows a clear cytoplasmic localization similar to GFP alone and is excluded from the chloroplasts (Fig. 4). In conclusion, PpHxk1 is a chloroplast-imported enzyme whose localization depends on an N-terminal transit peptide.

To confirm and further pinpoint the subcellular localization of PpHxk1, we isolated chloroplasts from wild type and hxl mutant protonemal tissue as described under “Experimental Procedures.” The purified chloroplasts were subjected to osmotic lysis and separated into soluble and membrane fractions by ultracentrifugation. The glucose-phosphorylating activity was then assayed in the crude extracts, in the purified chloroplasts, and in the chloroplast-soluble and membrane fractions. As shown in Fig. 5, we found that 80% of the total activity in crude extracts disappears in the hxl mutant, which is consistent with the results in Fig. 3. In the purified chloroplasts, PpHxk1 is further enriched, accounting for 85% of the glucose-phosphorylating activity. Interestingly, the PpHxk1-dependent activity is exclusively found in the soluble chloroplast fraction, indicating that PpHxk1 is a stromal enzyme. Furthermore, PpHxk1 seems to be the only glucose-phosphorylating enzyme in the chloroplast stroma because 98% of the stromal activity disappears in the hxl mutant (Fig. 5). In contrast, the much lower glucose-phosphorylating activity that is found in the chloroplast membrane fraction is not significantly affected in the hxl mutant, indicating that PpHxk1 does not contribute to this activity. We conclude that PpHxk1 is the major glucose-phosphorylating enzyme in chloroplasts and that it is a soluble enzyme, which is located in the chloroplast stroma.

The hxl Mutant Is Deficient in Glucose-induced Formation of Caulonemal Filaments—Since plant hexokinases have been implicated in glucose sensing and signaling (7, 21), we proceeded to investigate how the hxl mutant responds to externally added glucose. The formation of the Physcomitrella colony from regenerating protoplasts starts with a filamentous tissue type called protonemata (31). There are two protonemal cell types, chloronemal and caulonemal cells. The protonemata starts out as chloronemal filaments that eventually give rise to caulonemal filaments that produce side branches, some of which develop into buds, which in turn produce the gametophores, also known as leafy shoots. The transition between the cell types is influenced by auxin and cytokinins (32) and environmental factors such as light and nutrients (33, 34). It should
be noted that the two filamentous cell types differ in that chloronema contain more chloroplasts and are photosynthetically more active than caulonema. Caulonema are formed only if an adequate energy supply is available (35), and they help to spread the colony by their rapid radial growth. From a metabolic point of view, chloronema and caulonema can therefore be studied directly by knock-outs in the haploid gametophyte (22, 23). The gene, PpHXK1, does not have any close relatives in the Physcomitrella genome as revealed by our low stringency Southern blots. However, the finding of other hexokinase-encoding sequences such as PpHXK2 (Fig. 8) in our initial PCR shows that other more distantly related hexokinase genes exist in Physcomitrella. In this context, it should be noted that Arabidopsis has no less than six hexokinase encoding genes (16). Significantly, a targeted knock-out of the PpHXK1 gene revealed that it encodes the major hexokinase in Physcomitrella, which alone accounts for 80% of the glucose-phos-

## DISCUSSION

To investigate hexokinase function in plants, we have cloned a hexokinase gene from the moss *P. patens* where gene function can be studied directly by knock-outs in the haploid gametophyte (22, 23). The gene, *PpHXK1*, does not have any close relatives in the *Physcomitrella* genome as revealed by our low stringency Southern blots. However, the finding of other hexokinase-encoding sequences such as *PpHXK2* (Fig. 8) in our initial PCR shows that other more distantly related hexokinase genes exist in *Physcomitrella*. In this context, it should be noted that *Arabidopsis* has no less than six hexokinase encoding genes (16). Significantly, a targeted knock-out of the *PpHXK1* gene revealed that it encodes the major hexokinase in *Physcomitrella*, which alone accounts for 80% of the glucose-phos-
phorylating activity and 47% of the fructose-phosphorylating activity in protonemal tissue (Fig. 3). However, other enzymes must account for the residual activity in the mutant. It is likely that this involves the above mentioned additional hexokinases. This could also explain why the \textit{hxk1} mutant is viable and able to grow, although very poorly, on glucose in the dark (Fig. 6C).

We further found that the \textit{PpHxk1} enzyme is located in the chloroplast stroma (Fig. 5), a finding that is substantiated by our GFP fusion data, which also shows that this localization depends on the N-terminal 37 amino acid residues (Fig. 4). This finding prompted us to examine the N termini of other plant hexokinases. As seen in Fig. 8, plant hexokinase sequences fall into two distinct groups. One group is represented by spinach \textit{SoHxK1}, an enzyme for which the intracellular location has been determined (2). It has an N-terminal hydrophobic membrane anchor by which it is inserted into the outer envelope membrane of the chloroplast, facing the cytoplasmic side. Interestingly, 15 of those 25 plant hexokinases for which sequences are available have N termini with membrane anchors that are highly similar in sequence to \textit{SoHxK1} (Fig. 8). Therefore, it is probable that these enzymes also are targeted to the plastid outer envelope, although this remains to be proven. A common intracellular localization for this group of enzymes is supported by the TargetP program, which yields a high secretory pathway score for all but two of them (Fig. 8). However, this does not mean that they have to pass through this pathway, which is not how outer envelope proteins are thought to reach their destination (36, 37). Instead, the high secretory pathway score more likely reflects the presence of an N-terminal hydrophobic region, which is frequently found also in secretory pathway proteins, and the absence of a chloroplast transit peptide. With one known exception, \textit{Toc75} (38, 39), outer envelope proteins lack cleavable transit peptides and are instead inserted directly into the lipid bilayer (36, 37).

\textit{PpHxk1} clearly differs from these membrane-bound hexokinases. Its N terminus does not contain a hydrophobic membrane anchor. Instead, it is charged with several basic amino acid residues (Fig. 8) and as already discussed it is predicted to function as a chloroplast transit peptide. Some chloroplast transit peptides are followed by a luminal transfer peptide that directs the protein to the thylakoid lumen (36). An analysis using the SignalP program (30) failed to reveal the presence of a luminal transfer peptide in \textit{PpHxk1}, further supporting a stromal localization for the protein. We conclude that \textit{PpHxk1} is a novel type of chloroplast stromal hexokinase that differs from the above discussed membrane-associated enzymes. We will refer to this hexokinase as the Type A hexokinase, because it is responsible for most of the \textit{in vivo} enzyme activity, at least in \textit{Physcomitrella}, and to the membrane-associated enzyme represented by \textit{SoHxK1} as the Type B hexokinase.

An obvious question is whether Type A hexokinases are present in other plants and also whether \textit{Physcomitrella} has a Type B hexokinase. Sequence data suggest that both are true. Thus, we found that another \textit{Physcomitrella} gene, \textit{PpHXK2},
encodes a Type B hexokinase (Fig. 8). Conversely, nine sequenced hexokinases from *Arabidopsis*, potato, maize, and rice lack the highly conserved N-terminal sequence that is shared by the Type B enzymes (Fig. 8). Significantly, TargetP predicts that all of these hexokinases instead have N termini that are most likely to function as chloroplast transit peptides (Fig. 8). This finding suggests that chloroplast-imported Type A hexokinases are present also in other plants. It should be noted, however, that these hexokinases are more heterogeneous than the Type B enzymes in their N-terminal sequences (Fig. 8). Furthermore, the chloroplast transit peptide score is rather low in one case (At4g37840) and some of the Type A enzymes also contain putative membrane anchors, although they differ in sequence from those in the Type B enzymes. A non-chloroplast or non-stromal localization cannot therefore be ruled out for some of these proteins.

To determine how the two types of plant hexokinases are related to each other, we aligned all of the plant hexokinases for which full-length sequences are available. To avoid a bias based on the N-terminal sequences that we used to classify the hexokinases as Type A or B, the N termini were excluded as were the C termini where all of the sequences could not be aligned. The resulting core alignment was used to compute the dendrogram in Fig. 1. We found that the Type A enzymes are present within several deep branches in the tree, which suggests that they represent an ancestral form of plant hexokinase. This finding is consistent with the fact that membrane

**Fig. 6.** *PpHxk1* is required for glucose induction of caulonemal filaments and for normal growth on glucose in the dark. The colony morphologies of the wild type and *hxx1* mutant in the presence of different concentrations of glucose or mannitol (as an osmotic control) are shown. A, gametophore enhancement in the presence of 0.05 M glucose. *B*, induction of caulonemal filaments by 0.15 M glucose. C, growth in the dark with either 0.05 or 0.15 M glucose present in the plates. WT, wild type.

**Fig. 7.** RT-PCR analysis of *PpHXK1* expression under different conditions. Young protonemal tissue grown on cellophane was exposed to various treatments for 24 h as shown in the figure, after which the cells were harvested and total RNA was isolated. *PpHXK1* expression levels are shown as the relative amount obtained from 1 μg of total RNA as compared with an internal standard product (27). Intense light is continuous culture in 6000 lux. The error bars represent the mean ± S.D. from three independent samples.
anchored hexokinases have been found only in plants, indicating that the latter represent a later plant-specific innovation. Interestingly, we further found that the Type B enzymes form two distinct branches within the tree, indicating that hexokinases with N-terminal membrane anchors have arisen twice during plant evolution. One of these branches (Fig. 1, B1) is very ancient because its divergence from other plant hexokinases predates the separation of mosses from higher plants. This branch includes two predicted hexokinases from Arabidopsis and one from rice, none of which has been studied in any detail. The second branch (Fig. 1, B2) is of a more recent origin and includes the well studied hexokinases AtHxk1, AtHxk2, and SoHxk1 and their homologs in other plants. An obvious question is whether the acquisition of membrane anchors happened independently in the two branches. We think this is unlikely because the membrane anchors of the Type B1 and B2 hexokinases are very similar in sequence (Fig. 8). This similarity instead suggests that the membrane anchor originated once, probably within the older B1 branch, and then moved to an ancestor of the B2 enzymes by ectopic recombination.

We think that the Type A and B hexokinases have distinct physiological roles, which reflect their intracellular locations (Fig. 9). The Type B enzyme has already been proposed to function in supplying the cell with Glc-6-P during starch breakdown where it would help to maintain a concentration gradient that facilitates glucose export to the cytosol (2, 40). Obviously, the stromal Type A enzyme could not play such a role. However, it could function to make Glc-6-P directly available for further metabolism in the plastid after hydrolytic starch breakdown. There are several metabolic pathways in plastids, e.g. fatty acid synthesis and the pentose phosphate pathway, that use Glc-6-P. In the absence of a stromal hexokinase, glucose would have to be exported to the cytosol, phosphorylated, and then imported back into the chloroplast as Glc-6-P. According to this view, the Type A enzyme would be more important when the energy supply is limited, such as in sink tissues and during the night. This result is consistent with our finding that the hxl1 mutant has a particularly severe phenotype during growth in the dark (Fig. 6C). It is further conceivable that the Type A enzyme could function to phosphorylate glucose imported into the plastid by facilitated transport. Facilitated transport is important for glucose export during starch breakdown, but it can also occur in the other direction (40–43).
could provide an alternative route for supplying the plastid with Glc-6-P that would bypass the Glc-6-P/phosphate translocator (44). In this context, we note that the presence of hexokinases on both sides of the chloroplast envelope provides a possible mechanism for changing the direction and driving force of glucose transport by regulating the activities of the two hexokinases.

Several plant hexokinases have also been implicated in glucose sensing and signaling (16). It is notable, however, that these studies dealt exclusively with Type B enzymes, which are the only plant hexokinases that have been studied in some detail. This bias may reflect how many plant hexokinases were cloned, i.e. by complementation in yeast. We have found that PpHxk1 is unable to complement a yeast hxl hxb2 glk1 triple mutant (data not shown), and it is likely that other Type A hexokinases also would fail to do so because of the presence of the chloroplast transit peptide. Therefore, an important unresolved question is whether the Type A hexokinases participate in glucose sensing and signaling as has been proposed for the Type B enzymes (7, 21). The fact that PpHxk1 is located inside the chloroplast makes a direct role for this enzyme in cytoplasmic-nuclear signal transduction less likely than for the Type B enzymes. Our finding that glucose-induced caulonema formation is absent in the hxl mutant (Fig. 6B) could be taken as evidence of a glucose signaling function for PpHxk1, but it could also reflect a general effect of reduced glucose phosphorylation (18). Further experiments are needed to establish whether the Type A hexokinases have a role in signal transduction.

In conclusion, we have found that the PpHXX1 gene encodes a novel type of chloroplast stromal hexokinase that differs from the previously studied membrane-associated plant hexokinases. Furthermore, we found that this novel hexokinase is the major hexokinase in Physcomitrella, accounting for 80% of the glucose-phosphorylating activity and 47% of the fructose-phosphorylating activity. A knock-out mutant that lacks PpHxk1 is viable but has a complex phenotype that includes a failure to form caulonema filaments in the presence of glucose or sucrose and a severely reduced growth on these carbon sources in the dark. Sequence data suggest that most plants have both chloroplast-imported hexokinases similar to PpHxk1 and traditional membrane-bound hexokinases. Targeted knock-outs of additional hexokinase genes in Physcomitrella as well as other genes involved in sugar sensing or carbon metabolism may help to answer questions regarding hexokinase function not only in mosses but also in other plants.

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