Functional Characterization of Hexokinases in the Moss *Physcomitrella patens*

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Abstract

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Carbohydrates are important nutrients and structural components in all living organisms. In plants they affect the developmental and metabolic processes throughout the plant life cycle. However, the mechanisms by which plants recognise and respond to carbohydrates are mainly unknown. Hexokinase, an enzyme that mediates the first catalytic step in hexose metabolism, has recently been suggested to be involved in sugar sensing and signalling in plants.

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 to those in yeast. The aim of this thesis was to learn more about the function of plant hexokinases, both as key metabolic enzymes and in their putative role as sensors in sugar signalling, using *Physcomitrella patens* as a model system.

Five hexokinases from *Physcomitrella patens* were cloned and studied with respect to their subcellular localizations. PpHxk1 and PpHxk5 are located in the stroma of chloroplasts and are dependent on N-terminal transit peptides for correct localization. PpHxk2 and PpHxk3 both contain hydrophobic membrane anchors that localize the proteins to the outer envelope of chloroplasts. PpHxk4 contain neither a transit peptide nor an anchor, and is found in the cytosol.

A targeted knockout revealed that PpHxk1 is the major hexokinase in *Physcomitrella*, accounting for 80% of the glucose phosphorylating activity. Consistent with this, the knockout mutant exhibits an artificial starvation phenotype with altered sensitivities to plant hormones and a disturbed development.

Keywords: hexokinase, *Physcomitrella patens*, sugar signalling, subcellular localization, chloroplast, carbohydrate

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Contents

Appendix

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Olsson, T., Thelander, M. & Ronne, H. 2003. A novel type of chloroplast stromal hexokinase is the major glucose phosphorylating enzyme in the moss *Physcomitrella patens*. *Journal of Biological Chemistry 278*, 44439-44447.

II. Thelander, M., Olsson, T. & Ronne, H. 2005. Effect of the energy supply on filamentous growth and development in *Physcomitrella patens*. *Journal of Experimental Botany 56*, 653-662.

III. Olsson, T., Nilsson, A., Thelander, M. & Ronne, H. 2005. Structure and localization of hexokinases in the moss *Physcomitrella patens*. (Manuscript).

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Abbreviations

ABA	abscisic acid
ATP	adenosine triphosphate
cDNA	complementary DNA
CDS	coding sequence
DNA	deoxyribonucleic acid
EST	expressed sequence tag
GFP	green fluorescent protein
HXK	hexokinase
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcriptase PCR

Introduction

All organisms need to be able to balance the availability and requirement for carbohydrates in order to sustain their metabolism and support their growth and development. Plants are unique in that they produce the carbohydrates they require from light energy and carbon dioxide. This also makes them vulnerable. Plants are sessile and therefore utterly dependent on the sunlight in order to generate energy and various carbon compounds. Furthermore, not all tissues in the plant are able to utilize the light energy, but are instead dependent on the allocation of carbohydrates from other parts of the plant to meet their energy requirements. To cope with these problems, plants have developed a highly flexible metabolism and means to transport and store carbohydrates in anticipation of less favourable environmental conditions. The coordination of the carbohydrate partitioning and choice of developmental program demands that the plant has information regarding not only its carbohydrate and energy status but also where in the plant and for what purpose the available carbohydrates are needed. These requirements are met by a range of different signal molecules forming a complex network of signalling pathways within the plant. The exact nature of these pathways and the way that they interact with each other is still to a large extent unknown. However, one of the proposed players in this signalling network is hexokinase. It has been known as a key enzyme in the carbohydrate metabolism for many years, but has also recently been suggested to serve as a sugar sensor in plants. Still, the precise function of hexokinase in sugar sensing and signalling is yet to be elucidated.

Carbohydrate metabolism in plants

Plants capture the energy of sunlight and convert it into a usable form. This process is called photosynthesis and takes place in the chloroplasts. Chloroplasts are located in the cytosol and belong to an organelle family known as plastids. These organelles contain their own genome and are believed to have evolved from endosymbiotic cyanobacteria. However, most of the genes encoding chloroplast proteins reside in the nuclear genome. Such nuclear-encoded chloroplast proteins usually contain an N-terminal sequence, a targeting peptide, which targets them for import into the chloroplast (Keegstra & Cline, 1999). In addition to being essential for photosynthesis, plastids are important sites for biosynthesis of starch, fatty acids, and amino acids. Photosynthesis converts light energy to ATP and NADH, which in turn are used to reduce carbon dioxide into simple carbohydrates called triose phosphates. These triose phosphates can directly enter various metabolic pathways where they generate energy and provide building blocks for nucleic acids, proteins and fatty acids. Triose phosphates can also be converted to hexose phosphates, which are used for sucrose, starch and cell wall synthesis (Hopkins, 1995; Buchanan, Gruissem & Jones, 2000).

Sucrose synthesis and allocation

Not all organs or cells of a plant are able to carry out photosynthesis and through that fulfil their requirement for energy and carbon compounds. These organs are referred to as sink organs and are exemplified by the roots. The plant therefore needs to export energy from the photosynthesising cells, which are called source tissues, in order to support the sink tissues. The first step in this process is that triose phosphates are exported from the chloroplast to the cytosol, where they are used for hexose production and subsequent sucrose synthesis. Sucrose is a major end product of photosynthesis, and it is synthesised from UDP-glucose and fructose-6-posphate. Sucrose is a nonreducing sugar, and is therefore well suited as a transport compound from photosynthetic tissues to tissues in need of energy or tissues specialised for long-term storage.

Starch synthesis

A plant also has to be able to cope with fluctuations in the rate of photosynthesis. At night, there is no photosynthesis but the plant's need for energy and anabolic building blocks does not cease. Starch synthesis is a mechanism used to store excess photosynthate for later use. Starch synthesis in photosynthetic cells takes place during the day, when the rate of sucrose synthesis exceeds the rate of sucrose export from the cell. The excess of fixed carbon thus goes into starch synthesis. Starch is synthesised in the plastids and is also stored there. Temporary storage occurs in the chloroplasts, while long-term storage occurs in plastids specialised for that purpose, the amyloplasts. Much of the starch that is stored temporarily in the chloroplast is broken down during the night.

Utilization of stored energy

When a plant cell cannot meet its requirement for energy by increasing photosynthesis, either due to a dark period or because it is not a photosynthesising cell, it must rely either on sucrose transported from other tissues or starch that has been stored within the chloroplasts in order to obtain the needed energy and building blocks for anabolism.

Sucrose can be degraded either by sucrose synthase or invertase. The invertase reaction produces fructose and glucose, while the sucrose synthase reaction produces UDP-glucose and fructose. Starch on the other hand can be degraded either by phosphorylytic or hydrolytic cleavage reactions. The phosphorylytic degradation produces glucose-1-phosphate while the hydrolytic degradation produces glucose. The free hexoses that are the products of sucrose and hydrolytic starch degradation must be phosphorylated by a hexokinase before they can enter the hexose phosphate pool, and thus be of subsequent use in metabolic pathways.

Plants integrate the use of the glycolysis, the oxidative pentose phosphate pathway and various biosynthetic pathways in a unique way. Glycolysis converts hexoses into pyruvate and generates ATP and reductant (*e.g*. NADH). The main source of ATP production, however, is the Krebs cycle (in which the puryvate is

further converted into carbon dioxide and water) and oxidative phosphorylation. In plants, glycolysis takes place both in the cytosol and in plastids, either independently of each other or interacting across the plastid envelope (Plaxton, 1996), while the Krebs cycle and oxidative phosphorylation take place in the mitochondria. In addition to its role in ATP production, glycolysis also provides building blocks for anabolic reactions. One important building block is glucose-6-phosphate, which is used by the oxidative pentose phosphate pathway to generate reductants and pentose sugars for use in nucleic acid synthesis. Like the glycolysis, the oxidative pentose phosphate pathway occurs both in the cytosol and in plastids to different degrees, depending on the species and the stage of development (Neuhaus & Emes, 2000). Furthermore, the two pathways interact with each other in the sense that intermediates originating from one pathway can enter the other.

Hexokinases

The function and structure of hexokinases

Hexokinase catalyses the first step in glycolysis, the phosphorylation of hexoses. There is a distinction between glucokinases, fructokinases and hexokinases. Glucokinases exclusively phosphorylate glucose and fructokinases exclusively phosphorylate fructose, while hexokinases have dual specificity for both hexoses. It should be noted that in plants, there also exists fructokinases that belong to a protein family that is unrelated to the hexokinase family (Pego & Smeekens, 2000).

Most eukaryotes have multiple isoforms of hexokinase. *Saccharomyces cerevisiae* encodes three hexose phosphorylating enzymes; Hxk1, Hxk2 and Glk1 (Maitra & Lobo, 1983). The three yeast enzymes differ in their substrate specificites: Hxk1 is mainly a fructokinase, Glk1 a glucokinase, and Hxk2 (the major isozyme) an enzyme with dual specificity. Mammalian genomes encode one glucokinase and three hexokinases; type I, type II and type III. It should be noted, however, that the mammalian glucokinase is in fact an enzyme with dual specificity for glucose and fructose. This has led to the suggestion that this enzyme should be referred to as hexokinase IV, since glucokinase clearly is a misleading name (Cárdenas, Cornish-Bowden & Ureta, 1998). In plants, the *Arabidopsis thaliana* genome encodes six hexokinases (Frommer, Schulze & Lalonde, 2003) while the rice genome encodes ten hexokinases (unpublished observation).

The crystallization of yeast Hxk2 and human hexokinase I has revealed that the protein consists of one large and one small domain, between which the active site is situated (Aleshin *et al*., 2000; Kuser *et al*., 2000). The amino acid residues responsible for binding ATP and glucose have been identified in the crystal structures. Glucose is thought to bind first to the active site, which induces a slight conformational change that creates the binding site for ATP (Kuser *et al*., 2000).

Hexokinases from different species differ in molecular mass, generally ranging from 50-100 kDa. The mammalian hexokinases I, II and III fall into the 100 kDa group and consists of two halves whose sequences clearly are related to each other. These types of hexokinases are therefore thought have evolved by duplication and fusion of an ancestral gene (Cárdenas, Cornish-Bowden & Ureta, 1998). In contrast, the mammalian glucokinase (or hexokinase IV) is an unduplicated 50 kDa protein similar to the hexokinases found in most other eukaryotes. Finally, it should be noted that hexokinases are able to interact with each other to form dimers (Randez-Gil *et al*., 1998a; Frommer, Schulze & Lalonde, 2003).

The subcellular localization of hexokinases

The glucokinase and two hexokinases of *Saccharomyces cerevisiae* have been considered to be cytosolic, but recently Hxk2, which is required for glucose repression, has been shown to have a dual localization to both the cytosol and the nucleus (Randez-Gil *et al*., 1998b). The nuclear localization of Hxk2 is necessary for glucose repression of the *SUC2* gene, which encodes invertase (Herrero, Martínez-Campa & Moreno, 1998). Moreover, the nuclear localization is glucose regulated and dependent on a decapeptide in the N-terminal part of the protein, which in addition interacts with the Mig1 repressor (Ahuatzi *et al*., 2004).

The mammalian type I hexokinase is associated with the outer mitochondrial membrane, a localization which is dependent on a 15-amino acid N-terminal domain (Xie & Wilson, 1988; Gelb *et al*., 1992). The type I hexokinase also interacts with porin, also called VDAC, a protein that spans the outer membrane and serves as a membrane channnel for small metabolites including ATP (Felgner, Messer & Wilson, 1979; Lindén, Gellerfors & Nelson, 1982). Furthermore, the hexokinase preferentially binds to porins that are located in regions where the inner and outer mitochondrial membranes have close contact, where it favours ATP generated in mitochondrial respiration over cytosolic ATP (Wilson, 2003). The type II isozyme also contains a hydrophobic N-terminal sequence capable of targeting the enzyme to mitochondria but the protein has also been found in the cytosol (Sui & Wilson, 1997; Wilson, 2003). The type III isozyme lacks the N-terminal sequence and is found in the cytosol, particularly in the nuclear periphery (Preller & Wilson, 1992; Wilson, 2003). Finally, the mammalian glucokinase (hexokinase IV) has been reported to be associated with the actin cytoskeleton (Murata *et al*., 1997).

In plants, hexokinases have also been proposed to localize to various subcellular compartments (Figure 1). Most of the localization studies to date have been performed only by biochemical activity studies, without isolation and identification of the proteins involved. This means that fractions exhibiting predominantly fructose phosphorylating activity have been referred to as fructokinases while fractions exhibiting glucose phosphorylating activity have been designated as hexokinases. Thus, it remains unclear to what extent the various activities that have been described are mediated by true hexokinases or plant-specific fructokinases.

In spinach, two hexokinase and two fructokinase activities were initially found in leaves. One of the hexokinase activities was reported to be associated with mitochondria, while one of the fructokinase activities was found in the chloroplast stroma. The two remaining activities were suggested to be cytosolic (Schnarrenberger, 1990). Singh and colleagues (1993) could later show that there are two hexokinase-activity pools in spinach chloroplasts, where one was bound to the cytosolic side of the envelope and one resided in the chloroplast stroma. Wiese and colleagues (1999) cloned a hexokinase cDNA, and showed that the encoded protein, SoHxK1, is inserted into the outer envelope membrane of the chloroplasts. They further showed that this localization is dependent on a hydrophobic anchor at the N-terminus of the protein. The main role of SoHxK1 in both chloroplasts and plastids from non-green tissues is thought to be an immediate phophorylation of glucose that is exported from the chloroplasts as a product of hydrolytic starch breakdown. In castor oil seed, there are three hexokinase activities, where isozyme I is associated with the mitochondrial outer membrane, isozyme II is located in the plastid stroma, and isozyme III is cytosolic (Miernyk & Dennis, 1983). Hexokinase activity is also associated with mitochondria in both pea stems and leaves, while a fructokinase activity is found in the cytosolic fraction of the stems (Tanner, Copeland & Turner, 1983; Cosio & Bustamante, 1984). Hexokinase activity in seedling maize roots is distributed between cytosolic and non-cytosolic fractions. The non-cytosolic fraction includes mitochondria, microsomes and Golgi-derived vesicles. Additionally, at least two fructokinase activities are found in the cytosolic fraction. The non-cytosolic hexokinases in maize roots seem to be coupled to UDP-glucose formation, and might be involved in glycosylation reactions in the Golgi apparatus (Galina & da-Silva, 2000; da-Silva, Rezende & Galina, 2001).

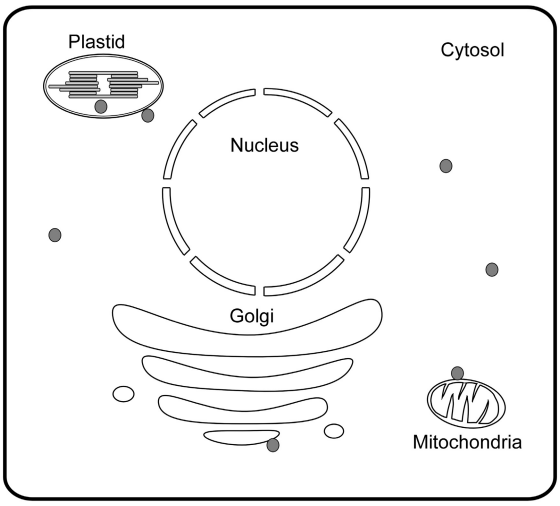

Figure 1. Proposed localizations of hexokinases in plant cells. Grey circles represent hexokinases.

A number of cDNAs and genes encoding hexokinases have been identified in different plants (Jang *et al*., 1997; Wiese *et al*., 1999, Veramendi *et al*., 1999; 2002), but only in a few cases, such as the above mentioned SoHxK1, has the subcellular location of the enzyme been determined. Most plant hexokinases that have been studied so far, including AtHxk1 and AtHxk2 (Jang *et al*., 1997) belong to the type B group (Olsson, Thelander & Ronne, 2003 [paper I]), which have N-terminal membrane anchors similar to that in SoHxK1. As discussed further below, we found that *Physcomitrella patens* also has another kind of hexokinase (type A), which is imported into the chloroplast stroma (Olsson, Thelander & Ronne, 2003 [paper I]) and we predicted that uncharacterized type A-like hexokinases in other plants also might be targeted to the chloroplast stroma. This prediction was recently confirmed by Giese and colleagues (2005) who found that a type A hexokinase in tobacco, NtHxk2, is localized in the plastid stroma. The expression of NtHxk2 is mainly restricted to tissues where starch turnover takes place, indicating that NtHxk2 might be involved in starch degradation.

Finally, in *Arabidopsis*, the gene products of *AtHXK2* and the putative hexokinase gene At1g50460, along with several other glycolytic enzymes, were reported to be associated with mitochondrial fractions, as determined from partial sequencing of peptides (Giegé *et al*., 2003). This has led to the suggestion that the entire glycolytic pathway is associated with mitochondria in plants, by attachment to the cytosolic face of the outer mitochondrial membrane. This micro-compartmentation of the glycolysis would allow pyruvate to be supplied directly to the mitochondria, where it subsequently is used as a substrate for the Krebs cycle.

Sugar sensing and signalling in plants

Sugars as signalling molecules

As described above, sugars are crucial as substrates in the carbon and energy metabolism and as structural components throughout a plant's life cycle. Accordingly, sugars have a major impact on plant growth and development. The developmental process is complex because of the multicellular nature of the plant. This requires extensive communication and coordination between different cells and tissues in the plant. Such intercellular communication is principally carried out by the plant hormones, such as abscisic acid, cytokinin, auxin, and ethylene (Hopkins, 1995). These hormones influence a wide variety of developmental and physiological processes ranging from seed development to senescence, stress responses and cell cycle completion. In addition, most of the above processes can be influenced by more than one plant hormone at a time.

In recent years, there has been increasing evidence that sugars also have important hormone-like functions that seem to be mediated both by modulation of plant hormone pathways, and by sugar-specific signalling pathways in plants (Rolland, Moore & Sheen, 2002). The fundamental role of sugars as signalling molecules has long been recognised in yeast, which utilizes a system that

optimises consumption of the preferred carbon source glucose. Thus, yeast exclusively ferments glucose when it is available. In the presence of glucose, genes that are needed for metabolism of other carbon sources are therefore transcriptionally repressed, while genes involved in uptake and fermentation of glucose are induced (Ronne, 1995; Johnston, 1999; Rolland, Winderickx & Thevelein, 2002).

Similar to microorganisms, plants also need to be able to adapt to variations in nutrient availability and energy status. Being multicellular, they also require mechanisms for resource allocation between source and sink tissues. It is established that photosynthesis is regulated by sugar in a feedback mechanism. Thus, when sugar concentrations increase there is repression of genes involved in photosynthesis and mobilization of stored reserves, while genes required for metabolism and storage are induced. The depletion of sugars leads to activation of photosynthetic genes and genes involved in carbohydrate mobilization (Koch, 1996). However, it is becoming increasingly clear that sugars do not only control the metabolism but are also involved in the regulation of numerous developmental processes throughout the plant life cycle, from germination to senescence (Rolland, Moore & Sheen, 2002; and references therein).

Sugar response screens

In order to identify components of sugar signalling pathways in plants the approach has been predominantly genetic, *i.e.* to isolate sugar response mutants. This has been accomplished by either of two major strategies. The first approach is based on the screening of mutagenized plant populations that carry a construct with a sugar responsive promoter in front of a reporter gene. This method was used to isolate *sucrose uncoupled* (*sun*) mutants (Dijkwel *et al*., 1997), *reduced sucrose response* (*rsr*) mutants (Martin *et al*., 1997), *impaired sucrose induction* (*isi*) mutants (Rook *et al*., 2001), the *low β-amylase* (*lba*) and the *high β-amylase* (*hba*) mutants (Mita *et al*., 1997a; 1997b). The second approach is based on the fact that some plants exhibit a sugar-dependent developmental arrest at the germination or seedling stage. There have been several screens performed for mutants that are able to overcome the inhibitory effect of high sugar levels on seedling development; mutants found in these screens include the *glucose insensitive* (*gin*, Zhou *et al*., 1998), *sugar insensitive* (*sis*, Laby *et al*., 2000), and *sucrose insensitive growth* (*sig*, Pego *et al*., 2000) mutants. The *mannose insensitive germination* (*mig*, Pego *et al*., 2000) mutants were similarly isolated in a screen based on the repression of seed germination by low levels of mannose. The principle of seedling developmental arrest has also been used to isolate mutants with enhanced sugar sensitivity, including *glucose super sensitive* (*gss*, Pego *et al*., 2000), *sucrose super sensitive* (*sss*, Pego *et al*., 2000), and *pleiotropic regulatory locus1* (*prl1*, Németh *et al*., 1998). It should be stated that all genes affected in the sugar response mutants are not necessarily involved in sugar signalling.

Hexokinase as a glucose sensor in plants

Hexokinase has in addition to its catalytic function in the metabolism also been implicated as a key player in plant sugar sensing and signalling. This notion originated from the yeast system where hexokinase long has been recognised as a part of the glucose repression machinery, but only recently has evidence for the exact role of the protein has begun to emerge in yeast (Moreno *et al*., 2005). In plants, experiments with different glucose analogues has demonstrated that only those analogues that can be used as substrates by hexokinase are able to modulate the expression of certain sugar regulated genes, including the chlorophyll a/b-binding protein (*CAB*) and ribulose-1,5-bisphosphate carboxylase small unit (*RBCS*) genes from *Arabidopsis* and the malate synthase (*MS*) and isocitrate lyase (*ICL*) genes from cucumber. Thus, mannose and 2-deoxyglucose, which both are phosphorylated by hexokinase, are able to repress sugar regulated genes, while 6-deoxyglucose and 3-*O*-methylglucose, which are taken up by the cell but not phosporylated by hexokinase, both are ineffective (Graham, Denby & Leaver, 1994; Jang & Sheen, 1994). Furthermore, hexokinase inhibitors are able to relieve the inhibitory effect of glucose on gene expression (Jang & Sheen, 1994). Finally, transgenic *Arabidopsis* plants expressing antisense constructs for hexokinase have been shown to be sugar hyposensitive, while plants overexpressing hexokinase are sugar hypersensitive. Thus, the antisense plants did not exhibit glucose-dependent repression of the *CAB* and *RBCS* genes, whereas the hexokinase overexpressors showed an enhanced repression of these genes (Jang *et al*., 1997).

These data support the notion that hexokinase could function as a sugar sensor in a sugar signalling pathway, although it is not an easy task to discriminate between the metabolic function and the proposed sensor function. This problem is illustrated by work in yeast, where the role of hexokinase in glucose repression is still not fully understood, even though the effect has been studied extensively by many research groups since its discovery 25 years ago (Entian, 1980). The role of plant hexokinases in a strict regulatory sensor function that is distinct from the catalytic activity has therefore been a subject of debate (Halford, Purcell & Hardie, 1999; Moore & Sheen, 1999). However, Moore and colleagues (2003) recently showed that *AtHXK1* mutants that lack detectable Hxk1 catalytic activity could still support various effects of glucose on gene expression and development, thus providing a strong indication that glucose signalling can be uncoupled from glucose metabolism in at least some cases.

Sugar signalling pathways

Studies of transgenic *Arabidopsis* plants suggest the presence of at least three separate glucose sensing and signalling pathways (Xiao, Sheen & Jang, 2000). The first is a hexokinase-dependent but glycolysis-independent pathway, which would reflect a distinct signalling function of hexokinase. The second is a hexokinase- and glycolysis-dependent pathway, which probably reflects the metabolic role of hexokinase, and the third is a hexokinase-independent pathway (Figure 2).

The hexokinase-dependent but glycolysis-independent signalling pathway controls the expression of genes involved in photosynthesis, such as the *Arabidopsis* chlorophyll a/b-binding protein (*CAB*), plastocyanin (*PC*), and ribulose-1,5-bisphosphate carboxylase small unit (*RBCS*). This pathway does not seem to be dependent on the catalytic activity of the hexokinase. This notion is supported by the fact that overexpression of *AtHXK1* enhances the repression of the above mentioned genes while no repression was seen in plants expressing *AtHXK1* antisense constructs (Xiao, Sheen & Jang, 2000). Significantly, overexpression of the major yeast hexokinase *ScHXK2* in *Arabidopsis* did not result in enhanced repression of the photosynthetic genes although the catalytic activity was elevated, indicating that the yeast enzyme was active (Jang *et al*., 1997). This indicated that yeast Hxk2 cannot substitute the AtHxk1 signalling function. Furthermore, the fact that 2-deoxyglucose is able to repress the expression of *CAB* while phosphorylated sugars are unable to do so indicates that further metabolism downstream of hexokinase is not needed for the control of expression of these genes (Jang & Sheen, 1994).

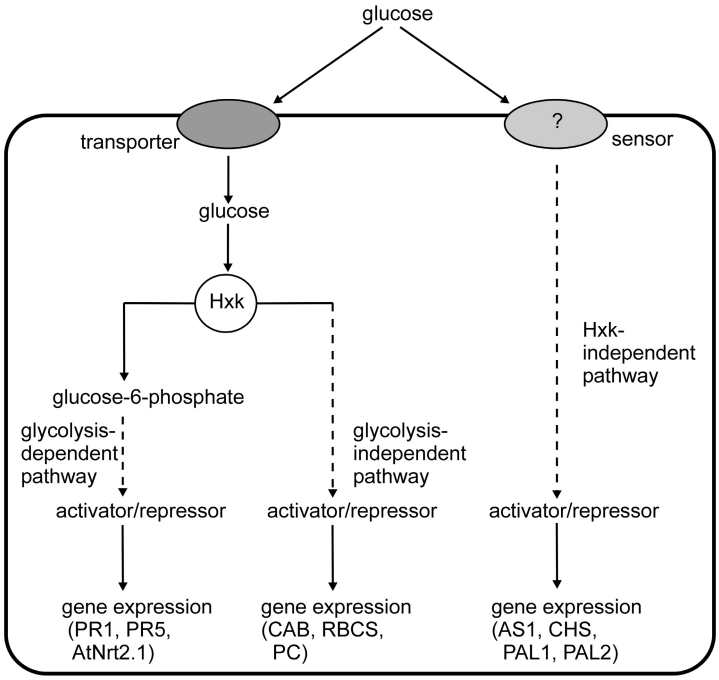

Figure 2. The three proposed glucose signalling pathways in plants.

The hexokinase- and glycolysis-dependent pathway regulates the expression of the pathogenesis-related genes *PR1* and *PR2* in *Arabidopsis*. In this case, overexpression of *ScHXK2* affects the induction of *PR1* and *PR5* to the same extent as overexpression of *AtHXK1* (Xiao, Sheen & Jang, 2000). Moreover, analysis of the expression of the sugar-inducible *Arabidopsis* nitrate transporter

gene *AtNrt2.1* revealed that mannose and 2-deoxyglucose, which are phosphorylated by hexokinase but not further metabolised, are unable to mimic the inducing effect of sucrose, glucose and fructose. Furthermore, the regulation of the gene is not altered in sugar sensing mutants, although the sugar induction of *AtNrt2.1* is abolished in an antisense *AtHXK1* line (Lejay *et al*., 2003). These data indicates that this pathway is dependent on the catalytic activity of hexokinase. The specific signal in this case may not be glucose itself but a metabolite downstream of hexokinase in the carbon metabolism.

It should further be noted that a knockout of the two redundant *Physcomitrella patens* genes *PpSNF1a* and *PpSNF1b*, which encode Snf1-related protein kinase 1, produces a phenotype which is largely opposite that of a *hxk1* knockout (Thelander, Olsson & Ronne, 2004). This raises the possibility that this kinase may function downstream of hexokinase in glucose signalling, as is the case for the Snf1 kinase in yeast (Ronne, 1995; Johnston, 1999; Rolland, Winderickx & Thevelein, 2002). The *snf1* and *hxk2* knockout mutants have opposite phenotypes in yeast, which reflects the fact that a hexokinase-dependent signal inhibits the Snf1 kinase within the glucose repression pathway.

The hexokinase-independent pathway, finally, regulates genes such as asparagine synthase (*AS1*), phenylalanine ammonia-lyase (*PAL1* and *PAL2*), and chalcone synthase (*CHS*). This pathway is not at all affected by changes in the catalytic activity of hexokinase. Furthermore, the effect is triggered also by the glucose analogues 6-deoxyglucose and 3-*O*-methylglucose, which are not substrates for hexokinase (Xiao, Sheen & Jang, 2000).

It should be mentioned that there also are indications of a sucrose-specific sensing and signalling pathway (Chiou & Bush, 1998; Tiessen *et al*., 2003). Because of the degradation of sucrose into glucose and fructose, it can be complicated to decide which molecule is responsible for the signalling event. However, in some cases, it could be shown that fructose and glucose were unable or less effective than sucrose in triggering responses, which provides evidence that the hexoses are not involved in this signalling pathway. This also indicated that further metabolism of sucrose is not necessary for the signalling event to take place. Interestingly, the Snf1-related protein kinase 1 has also been suggested to be involved in a sucrose-specific signalling pathway, but in this case independently of hexokinase (Tiessen *et al*., 2003).

Sugar and hormone cross-talk

Interestingly, the characterization of the mutants isolated in the sugar screens described above has led to the identification of mutations in genes previously implicated in plant hormone biosynthesis or signalling, thus providing a link between sugar and hormone signalling pathways. Accordingly, the sugar resistant mutants *sis4*/*gin1*/*isi4* (Laby *et al*., 2000; Zhou *et al*., 1998; Rook *et al*., 2001) have been shown to be allelic to *aba2* (Léon-Kloosterziel *et al*., 1996). The *GIN1*/*ABA2* gene was subsequently cloned, and shown to encode a short-chain dehydrogenase/reductase involved in the synthesis of ABA (Cheng *et al*., 2002).

Furthermore, the sugar resistant mutant *gin5* (Arenas-Huertero *et al*., 2000) is allelic to the ABA biosynthesis mutant *aba3* (Léon-Kloosterziel *et al*., 1996) and the sugar resistant mutants *sis5*/*gin6*/*isi3*/*sun6* (Laby *et al*., 2000; Arenas-Huertero *et al*., 2000; Rook *et al*., 2001; Huijser *et al*., 2000) are allelic to *abi4*, which is an ABA insensitive mutant (Finkelstein *et al*., 1998).

There is also evidence of cross-talk with the ethylene response pathway. Thus, the *sis1*/*gin4* mutants (Gibson, Laby & Kim, 2001; Rolland *et al*., 2002) are allelic to the ethylene constitutive mutant *ctr1*. Furthermore, the ethylene overproducer mutant *eto1* is sugar resistant while the ethylene insensitive mutants *etr1* and *ein4* are hypersensitive to sugar (Gibson, 2004). The stability of a key transcriptional regulator in ethylene signalling, Ein3, has been shown to be enhanced by ethylene and reduced by glucose (Yanagisawa, Yoo & Sheen, 2003). Finally, an *AtHXK1* null mutant was shown to be insensitive to auxin and hypersensitive to cytokinin, thus demonstrating an interaction between the AtHxk1-dependent glucose signalling pathway, and the auxin and cytokinin response pathways (Moore *et al*., 2003).

In conclusion, there is evidence of extensive cross-talk between the sugar sensing and plant hormone signalling pathways. A major challenge in plant biology is therefore to determine to what extent sugars exert their effects through unique pathways that respond only to specific sugars, or by modulating plant hormone pathways. Studies of knockout mutants in *Physcomitrella patens* and their phenotypes may help to solve this important question.

The moss *Physcomitrella patens*

Functional genomics in plants

Functional genomics, *i.e.* designating each gene in a genome a precise function, is one of the fundamental areas of plant biology today. There are several approaches for revealing gene functions. The classical forward genetics approach is based on the phenotypic screening of plants that have been mutagenized by chemical or physical treatments. The disadvantage of this method is the laborious positional cloning of the mutated gene locus once an interesting phenotype has been found. The most frequent reverse genetics approach used to perform large-scale functional genomics in plants has been to disrupt genes through random integration by introducing either *Agrobacterium*-derived T-DNA (Krysan, Young & Sussman, 1999) or a transposon (Martienssen, 1998) into the plant. Alternatively, knock-down methods such as antisense RNA and RNAi can be used to prevent expression of the gene of interest (Horiguchi, 2004). A very important method in reverse genetics is gene targeting, *i.e.* the generation of specific mutations in the genome by homologous recombination mediated integration of foreign DNA sequences. The possibility to specifically knock out a gene of interest is what has made the yeast *Saccharomyces cerevisiae* such a powerful model system (Struhl, 1983). Unfortunately, a targeted knockout of a particular gene in a flowering plant is almost impossible because homologous recombination occurs only at very low frequencies (Mengiste & Paszkowski, 1999). The

functional characterization of particular genes in plants has therefore been performed either by transposon mutagenesis, overexpression or antisense RNA suppression.

Physcomitrella *as a plant model system*

The bryophyte *Physcomitrella patens* was originally chosen as a model system to study developmental processes in plants due to its simple morphology (Cove *et al*., 1991 and references therein; Cove & Knight, 1993). A very significant turn of events, however, was the discovery that *Physcomitrella* has an efficient native system for homologous recombination (Schaefer & Zrÿd, 1997; Schaefer, 2001) in contrast to other land plants, which makes knockouts possible. In addition, the dominating growth phase of *Physcomitrella* is haploid, which makes it possible to study knockouts and other recessive mutations without any backcrossing to obtain homozygous diploids. These two features make *Physcomitrella* an ideal model system for studying plant gene function by means of reverse genetics.

Physcomitrella is fast growing and is easily maintained both in liquid and on solid media. The cultures can be kept for several months in the fridge, and long-term storage of *Physcomitrella* strains by cryopreservation has been shown to be reliable (Schulte & Reski, 2004). Furthermore, dried spores can be stored for several years (Frank, Decker & Reski, 2005). Several important molecular biology techniques have now been established in *Physcomitrella*. These include gene-trap and enhancer-trap systems (Nishiyama *et al*., 2000; Hiwatashi *et al*., 2001), RNA-interference (Bezanilla, Pan & Quatrano, 2003), and a system to identify protein phosphorylation (Heintz *et al*., 2004). Transformation can be accomplished by either PEG-mediated DNA uptake by protoplasts (Schaefer *et al*., 1991) or particle bombardment (Bezanilla, Pan & Quatrano, 2003).

Many developmental and regulatory processes in *Physcomitrella* seem to be influenced by the same factors as in higher plants. These factors include light (Imaizumi *et al*., 2002), calcium (Tucker *et al*., 2005), gravity (Reski, 1997) and phytohormones such as auxin (Ashton, Grimsley & Cove, 1979), cytokinin (Ashton, Grimsley & Cove, 1979; von Schwartzenberg *et al*., 1998), and abscisic acid (Knight *et al*., 1995; Nagao *et al*., 2005). The mechanisms through which these effectors operate seem to be fairly conserved in some cases, such as the response to light which is mediated through cryptochrome, red-light receptor, dichroic phytochrome and phototropin in *Physcomitrella* (Uenaka, Wada & Kadota, 2005). In other cases, such as the response to abiotic stresses, there seem to be conserved mechanisms (Knight *et al*., 1995) as well as pathways that have been altered during evolution (Kroemer, Reski & Frank, 2004).

Physcomitrella is also interesting from an evolutionary developmental genetics perspective. The last common ancestor of bryophytes and seed plants lived about 450 million years ago (Theissen *et al*., 2001). The phylogenetic position of mosses near the base of land plants makes *Physcomitrella* ideal for addressing the question on of how the land plants have evolved.

The Physcomitrella life cycle

The life cycle of *Physcomitrella* is typical of a moss, in that it comprises the alteration of two generations, the haploid gametophyte and the diploid sporophyte. The dominant haploid gametophyte consists of filamentous protonemal tissue and upright gametophores, also called leafy shoots (Figure 3).

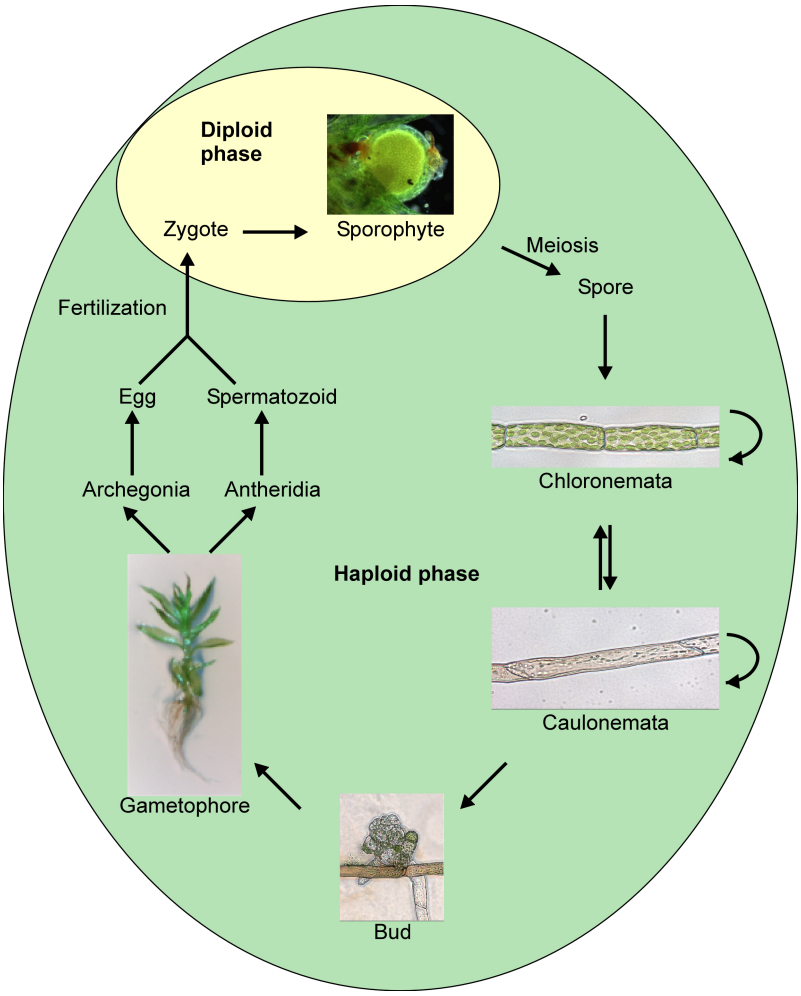

Figure 3. The life cycle of *Physcomitrella patens*.

The development of *Physcomitrella*, which has been described by Reski (1997), starts as a haploid spore germinates and grows out to form protonemal filaments. These filaments consist of two distinct cell types, chloronemata and caulonemata, which grows by apical cell divisions and sub-apical branching. Chloronemal cells are characterized by cell walls that are perpendicular to the growth axis, and have numerous round chloroplasts. Caulonemal cells, in contrast, have oblique cell walls and contain fewer and smaller plastids. Additionally, caulonemal cells are usually longer than chloronemal cells and have a higher growth rate. The first cell type to form from the germinating spore is chloronemal. The next differentiation step, the transition of chloronema to caulonema is induced by auxin (Ashton, Grimsley & Cove, 1979), and it takes several cell divisions before the transition is complete.

In contrast to side branches from chloronemal filaments which only can develop into a new chloronemal filament, caulonemal side-branch initials can have one of three fates: develop into a chloronemal side branch, a caulonemal side branch or develop into a bud. The bud is the initial of the upright gametophore, which consists of a stem and one cell-layer thick leaves. The stem contains hydroids, which are specialised conducting cells, although not a true vascular system. From the base of each gametophore root-like rhizoids protrude. The rhizoids are morphologically similar to caulonemal cells but do not branch and appear to serve mainly as a structural support for the gametophore. The gametophore bears the sex organs. *Physcomitrella* is monoecious, which means that both male (antheridia) and female (archegonia) sex organs develop on the same gametophore. Fertilization requires a surface water film in order for the male gametes (spermatozoids) to be able to swim down the neck of the archegonia. The zygote develops into an embryo, which grows out to become the next generation, the diploid sporophyte. Within the spore capsule the diploid cells then undergo meiosis and produce around 5000 haploid spores, thus completing the life cycle.

Physcomitrella genomics and transcriptome

The haploid genome size of *Physcomitrella patens* is estimated to be around 511 Mbp (Schween *et al*., 2003) distributed on 27 chromosomes (Reski *et al*., 1994). The genome of *Physcomitrella* is currently being sequenced by the Joint Genome Institute (JGI) and unassembled sequence data is already available at http://www.jgi.doe.gov. The first draft of the complete genome is expected to be released by the end of the year. This will make *Physcomitrella* the fourth plant genome sequenced after *Arabidopsis*, rice and poplar (Reski, 2005).

Even though information on the genome organisation is still missing, there is much information on the *Physcomitrella* transciptome available. Over 100 000 public ESTs (Nishiyama *et al*., 2003) and an additional of 110 000 proprietary ESTs (Rensing *et al*., 2002) are accessible in different databases representing more than 95% of the *Physcomitrella* transcriptome. In a recent report by Rensing and colleagues (2005) all EST and CDS data available were clustered to produce a genome-wide analysis of protein encoding genes in *Physcomitrella*. This resulted in the prediction of in total 19081 non-redundant ORFs. Approximately 30% of

these putative transcripts have a homolog in both the rice and *Arabidopsis* transcriptome, while 134 transcripts are not present in seed plants but can be found in other kingdoms. The fraction of gene products associated with metabolism is significantly higher in bryophytes than in seed plants (Lang *et al*., 2005). The organisation of exons and introns are generally conserved between *Physcomitrella* and *Arabidopsis* (Rensing *et al*., 2004; Champagne & Ashton, 2001), although the *Physcomitrella* introns seem to be longer than those in *Arabidopsis* (Harries, Pan & Quatrano, 2005; Rensing *et al*., 2005). *Physcomitrella* has been suggested to have smaller gene families than *Arabidopsis* (Rensing *et al*., 2002), however this is contradicted by our finding that there seems to exist 11 hexokinase genes in *Physcomitrella* compared to six in *Arabidopsis* (unpublished results).

Aims of the present study

Carbohydrates are important nutrients and structural components in all living organisms. In plants they affect the developmental and metabolic processes throughout the plant life cycle. Hexokinase mediates the first catalytic step, sugar phosphorylation, in hexose metabolism, but has also been implicated in carbohydrate sensing and signalling. Still, the mechanisms by which plants recognise and respond to carbohydrates are mainly unknown. The aim of the present study was to gain more insight in the function of hexokinases in plants, both as key metabolic enzymes and in their putative role as glucose sensors in the sugar signalling system.

Results and discussion

A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens* (I)

In order to study the function of hexokinases in *Physcomitrella patens*, we used PCR techniques to clone a full length cDNA and the corresponding gene, which was named *PpHXK1*.

Several genes from the *Physcomitrella* genome have been shown to have the same exon/intron organization as higher plants (Schaefer, 2002). Consistent with this we found that the exon/intron distribution of *PpHXK1* is identical to that of the *Arabidopsis AtHXK2* gene (Jang *et al*., 1997). A low stringency Southern blot indicated that there are no other genes that are very closely related to *PpHXK1* in the *Physcomitrella* genome, although an additional weak band showed the existence of at least one more distant relative. This is also consistent with our recovery of other hexokinase encoding sequences in the initial PCR. The expression of *PpHXK1* is essentially constitutive in young protonemal tissue, which was shown using quantitative RT-PCR.

The Hxk1 protein was predicted by the TargetP software (Emanuelsson *et al*., 2000) to be chloroplast-imported, with a possible N-terminal transit peptide cleavage site after amino acid residue 37 (Nielsen *et al*., 1997). We could show that Hxk1 indeed localizes to the chloroplasts by making translational fusions with GFP. While GFP alone localizes to the nucleus and cytoplasm, the Hxk1-GFP fusion is exclusively found in the chloroplasts. This localization is dependent on the N-terminal transit peptide, since a truncated Hxk1-GFP fusion lacking the signal peptide is excluded from the chloroplasts and shows a clear cytoplasmic localization similar to that of GFP alone.

We proceeded to knock out the *PpHXK1* gene, utilizing the fact that gene targeting in *Physcomitrella* works with frequencies comparable to those in yeast (Schaefer & Zrÿd, 1997; Schaefer, 2001). Interestingly, enzymatic studies revealed that the *hxk1* knockout mutant has a strongly reduced ability to phosphorylate glucose, only 22% of the wild type activity remained. We also tested the fructose phosphorylating activity since many hexokinases have a dual specificity for glucose and fructose. The *hxk1* mutant exhibited 53% of the wild type fructose phosphorylating activity. We could conclude that Hxk1 has a dual specificity for glucose and fructose.

In order to further pinpoint the subcellular localization of Hxk1 we purified chloroplasts from both *Physcomitrella* wild type cells and the *hxk1* mutant. The glucose phophorylating activity was examined in both membrane and stromal fractions. We could show that the PpHxk1-dependent glucose phosphorylating activity is exclusively found in the soluble fraction, demonstrating that the enzyme is imported into the stroma of the chloroplasts. It seems to be the only glucose phosphorylating enzyme there since 98% of the activity disappears in the *hxk1* knockout mutant. The much lower activity in the membrane fraction is not affected in the hxk1 mutant, which indicates that Hxk1 does not contribute to this activity. We suggested at that time that this activity might be due to another hexokinase-encoding gene named *PpHXK2*, found in the initial PCR. PpHxk2 has a predicted membrane anchor similar to that of spinach SoHxk1, which has been shown to be inserted into the outer envelope membrane of the chloroplast, facing the cytoplasmic side (Wiese *et al*., 1999). We were subsequently able to confirm that PpHxk2 (and also a related protein, PpHxk3) localizes to the chloroplast envelope (see paper III below).

We proceeded to study how the *hxk1* mutant responds to externally added sugar since hexokinases have been implicated to function in glucose sensing and signalling (Jang *et al*., 1997; Moore *et al*., 2003). We found that wild type moss colonies respond to 0.05 M externally added glucose by enhancing the formation of gametophores whereas the addition of 0.15 M glucose enhances the formation of caulonemal filaments protruding from the edge of the colony. In the *hxk1* mutant the enhancement of gametophores at 0.05 M glucose is unaltered but the mutant is deficient in the response to 0.15 M glucose.

The finding of a chloroplast targeting peptide in PpHxk1 prompted us to compare the N- termini of all plant hexokinase sequences that were available at that time. The sequences fell into two distinct groups, which we called Type A and Type B. The Type A hexokinases all have predicted chloroplast transit peptides, similar to PpHxk1. Type B are hexokinases with N-terminal hydrophobic membrane anchors, as exemplified by SoHxk1 (Wiese *et al*., 1999). The type B group comprises all those hexokinases from higher plants that so far has been studied in detail (Wiese *et al*., 1999; Jang *et al*., 1997; Veramendi *et al*., 1999; 2002). It should be noted, however, that the sequencing of more plant hexokinase genes has since revealed a more complicated picture. Thus, we found a hexokinase, PpHxk4, that lacks N-terminal extension (see paper III below) and

some other plant hexokinases have N-terminal extensions that resemble neither the Type A nor the Type B enzymes.

We next investigated how the different types of hexokinases are related to each other. An evolutionary tree was computed from the core sequences of the hexokinases in order to avoid any bias derived from the N-termini. We found that the Type A enzymes are present in several deep branches within the tree, indicating that they represent an ancestral form of plant hexokinase. The Type B enzymes form two distinct branches, which we named B1 and B2. The B1 branch appears to be very ancient since it diverged from other plant hexokinases before the separation of mosses from higher plants. In contrast, the B2 branch appears to be of more recent origin and might have arisen through an ectopic recombination event that copied the membrane anchor from a Type B1 enzyme to the ancestor of the Type B2 enzymes.

We think that the two types of hexokinases have distinct physiological roles, which reflect their intracellular locations. The Type B enzyme has been proposed to function in supplying the cell with glucose-6-P during starch breakdown, where it would help to maintain a concentration gradient over the plastid membrane that facilitates glucose export to the cytosol (Weber *et al*., 2000). The Type A enzyme could function to make glucose-6-P directly available for further metabolism in the plastid after hydrolytic starch breakdown. The Type A enzyme could also function to phosphorylate glucose imported into the plastid by facilitated transport. 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.

Effect of the energy supply on filamentous growth and development in *Physcomitrella patens* (II)

Our initial characterization of the *Physcomitrella hxk1* knockout mutant revealed that 0.15 M glucose stimulates caulonema formation in light grown wild type colonies and that this effect is reduced in the mutant, suggesting a link between developmental regulation and the energy supply. This prompted us to investigate the developmental response of the *hxk1* mutant to different growth conditions.

We found that the *hxk1* mutant generally has a somewhat smaller colony diameter than the wild type, indicating a reduced growth rate. The *hxk1* mutant responds normally to auxin while it is hypersensitive to cytokinin. Furthermore, the *hxk1* mutant shows a clear hypersensitivity to the inhibitory effect of abscisic acid on colony size. Light microscopy analysis of chloronemal cells grown under standard conditions further showed that the *hxk1* mutant cells have a somewhat swollen appearance compared to the wild type. Abscisic acid is known to cause a change in cell shape resulting in thicker and less elongated protonemal cells, known as brood cells (Goode, Stead & Ducket, 1992) and consistent with this, the mutant phenotype is more pronounced in the presence of ABA. This prompted us to investigate if the *hxk1* mutant had elevated endogenous ABA levels, but this

could not be seen under any conditions tested. We concluded that the cell-shape phenotype of *hxk1* mutant is not mediated by changes in endogenous ABA levels.

Next, we investigated how the formation of caulonemal filaments is affected in the mutant and under different growth conditions. It has previously been reported that auxin stimulates and cytokinin inhibits the formation of caulonemal filaments under high light conditions (Ashton, Grimsley & Cove, 1979; Reski, 1998). We observed that both light and external glucose strongly enhance the formation of caulonema in the wild type, while in the *hxk1* mutant the basal rate of caulonema formation is reduced several-fold irrespective of the growth conditions.

We proceeded to examine chloronemal branching in the mutant and the wild type. We found that both high light and auxin reduce chloronemal branching while cytokinin stimulates it in the wild type. The effect on side branch length is less pronounced although a slight inverse correlation with the branching frequency is seen. Glucose has a more complex effect in that it reduces the chloronemal branching frequency but increases the number of cells per chloronemal side branch. In the *hxk1* mutant the number of branches per chloronemal cell is enhanced under all conditions tested, while the lengths of the branches are generally shorter. The increase in branch length in response to glucose which is seen in the wild type is blocked in the *hxk1* mutant. The effects on caulonema formation and chloronemal branching are opposite for all the conditions that we tested, which suggests a balance between the two filament types. PpHxk1 is the major glucose phosphorylating enzyme in *Physcomitrella* protonemal tissue. The *hxk1* mutation is therefore likely to cause a significant reduction in hexose phosphorylation, producing less ATP and therefore creating a condition of artificial starvation. We therefore interpreted our findings as evidence that the available energy supply affects the balance between chloronemal and caulonemal cell types.

We could also show that the *hxk1* mutation causes buds to appear on chloronemal filaments. This is rarely seen in the wild type but can be induced by externally added cytokinin (Reski & Abel, 1985). The *hxk1* mutant has a significant number of chloronemal buds under normal growth conditions and the number of chloronemal buds is not further increased in the presence of cytokinin. Auxin completely blocked the formation of buds, as shown previously by Ashton, Grimsley & Cove (1979). We concluded that the developmental program that controls bud formation is disturbed in the mutant and that cytokinin and hexokinase may affect bud formation through a common mechanism.

Structure and localization of hexokinases in the moss *Physcomitrella patens* (III)

Our finding that PpHxk1 is located in the stroma of chloroplasts encouraged us to investigate where other hexokinases in *Physcomitrella* are localized. We started out by cloning full-length cDNAs of four additional hexokinases using PCR primers obtained from hexokinase-like sequences found in the publicly available

Physcomitrella EST database (Nishiyama *et al*., 2003). We also cloned the corresponding genes, which we called *PpHXK2*, *PpHXK3*, *PpHXK4* and *PpHXK5*.

In order to see how the different hexokinases are related to each other we constructed an evolutionary tree in the same manner as in (I) excluding the N- and C-termini to avoid bias. The tree was computed using only *Arabidopsis* and rice sequences along with our cloned *Physcomitrella* hexokinases because both these genomes have been fully sequenced and we believed this would provide a more complete picture of the plant hexokinase family. Surprisingly, we found that all *Physcomitrella* hexokinases clustered in one single branch in the tree, while the rice and *Arabidopsis* hexokinases were more widely dispersed throughout the tree.

We next investigated the subcellular localization of our new hexokinases. Both PpHxk2 and PpHxk3 belong to the Type B hexokinases described earlier in (I), since they both contain a predicted N-terminal membrane anchor. By making translational GFP-fusions, we could show that both PpHxk2 and PpHxk3 are localized to the chloroplast envelope and that this localization is dependent on the membrane anchors. Furthermore, they both also localize to small ring-like structures within the cells. However, we suspect that these might be artefacts due to overexpression.

PpHxk4 appears to represent a novel type of hexokinase, which we termed Type C. It neither has a signal peptide nor a membrane anchor, and is more distantly related to the other *Physcomitrella* hexokinases based on its core sequence. We expected this protein to be cytosolic because of the absence of a signal peptide. GFP-fusions showed that the protein is indeed cytosolic but in addition to this also localizes to the nucleus.

PpHxk5 contains a predicted N-terminal transit peptide and thus clearly belongs to the Type A hexokinases. GFP-fusions confirmed that the protein has a localization similar to PpHxk1, *i.e.* it is imported into the chloroplasts. As for PpHxk1, this localization was also shown to be dependent on the N-terminal signal peptide.

Several plant hexokinases of the Type B group were originally cloned by complementation of a hexokinase-deficient yeast strain (Jang *et al*., 1997; Veramendi *et al*., 1999; 2002). We had previously noted that PpHxk1 is unable to complement a yeast *hxk1 hxk2 glk1* triple mutant and we reasoned that the chloroplast transit peptide might interfere with the folding or targeting in yeast. However, we found that PpHxk1 lacking the transit peptide is still unable to complement a yeast triple mutant. In contrast, PpHxk3 is able to complement a yeast triple mutant, which is in agreement with the complementation cloning of other Type B hexokinases (Jang *et al*., 1997; Veramendi *et al*., 1999; 2002).

Conclusions

The *Physcomitrella patens* gene *PpHXK1* encodes a novel type of chloroplast stromal hexokinase, which is dependent on its N-terminal transit peptide for correct localization.

Hxk1 accounts for most of the glucose phosphorylating activity in young protonemal tissue.

A targeted knock out of the *PpHXK1* gene revealed an artificial starvation phenotype. Furthermore, the *hxk1* mutation causes buds to appear on chloronemal filaments and confers hypersensitivity to abscisic acid and cytokinin.

Both PpHxk2 and PpHxk3 are Type B hexokinases and localize to the outer envelope of the chloroplast.

PpHxk4 is a novel type of hexokinase, without targeting peptide or membrane anchor, which localizes to the cytosol and to the nucleus.

PpHxk5 is a Type A hexokinase, which localizes to the stroma of chloroplasts similar to PpHxk1.

Future perspectives

Hexokinase has long been considered as an unexciting enzyme at the start of glycolysis. However, the realisation that hexokinases are involved not only in metabolic processes, but also many essential developmental processes during the plant life cycle, and that there are several isozymes localized to different cellular compartments, has changed this opinion. The complete picture including the number of hexokinases, their localization and precise functions, is not known for any plant. Using *Physcomitrella* as a model system can help to elucidate the full story of hexokinase function in plants. There are several directions that should be followed.

In order to reveal the complete picture we first need to know the number of hexokinases in the *Physcomitrella* genome. This is a quite simple task to complete once the genome is fully sequenced. Using the sequences already available from the genome project we have identified in total 11 hexokinases in *Physcomitrella*. This might be troublesome news for a knockout strategy but actually need not to be. So far, only five of these hexokinases have been found in the available EST libraries, which might mean that not all of them are expressed, at least not in the cell types used for making the libraries or under the conditions used for cultivation. There might be several pseudogenes if the theory of unusually frequent gene duplications is true. The hexokinases also seem to be localized to

very different compartments in the cell. These questions of whether they all are expressed and where they localize to are important to answer.

Obviously, the gene targeting technique should be utilized on selected hexokinase genes. This will without a doubt give insights in the functions of the different hexokinases through studies of the resulting phenotypes. The resulting knockout mutants can also be used in hexokinase enzyme activity studies. This will demonstrate if all hexokinases are catalytically active. Moore and colleagues (2003) have proposed that the catalytic activity of AtHxk1 can be uncoupled from the sensing activity. Might some hexokinases have lost all their catalytic activity in favour of a signalling function? In this context, it should be noted that there are glucose transporters in yeast (Snf3 and Rgt2) that have lost their transporter activities and instead function as glucose sensors (Johnston, 1999; Rolland *et al*., 2002).

Utilising a *Physcomitrella* yeast two-hybrid library already available in our lab to fish for hexokinase interacting partners, might give us the means to find genetic evidence both for a hexokinase-mediated sugar sensing pathway and for cross talk between this pathway and the pathways of different plant hormones, for example that of ABA.

References

Ahuatzi, D., Herrero, P., de la Cera, T. & Moreno, F. 2004. The glucose-regulated nuclear localization of hexokinase 2 in *Saccharomyces cerevisiae* is Mig1-dependent. *The Journal of Biological Chemistry 279*, 14440-14446.

Aleshin, A.E., Kirby, C., Liu, X., Bourenkov, G.P., Bartunik, H.D., Fromm, H.J. & Honzatko, R.B. 2000. Crystal structures of mutant monomeric hexokinase I reveal multiple ADP binding sites and conformational changes relevant to allosteric regulation. *Journal of Molecular Biology 296*, 1001-1015.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J. & Léon, P. 2000. Analysis of Arabidopsis glucose insensitive mutants, *gin5* and *gin6*, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. *Genes and Development 14*, 2085-2096.

Ashton, N.W., Grimsley, N. H. & Cove, D. J. 1979. Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta 144*, 427-435.

Buchanan, B., Gruissem, W. & Jones, R. 2000. Biochemistry and molecular biology of plants. Courier Companies, Inc. *American Society of Plant Physiologists, United States of America*.

Cárdenas, M.L., Cornish-Bowden, A. & Ureta, T. 1998. Evolution and regulatory role of the hexokinaes. *Biochimica et Biophysica Acta 1401*, 242-264.

Champagne, C. & Ashton, N. 2001. Ancestry of KNOX genes revealed by bryophyte (*Physcomitrella patens*) homologs. *New Phytologist 150*, 23-26.

Cheng, W.H., Endo, A., Zhou, L., Prenney, J., Chen, H.C., Arroyo, A., Léon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T. & Sheen, J. 2002. A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *The Plant Cell 14*, 2723-2743.

Chiou, T.J. & Bush, D.R. 1998. Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Sciences of the USA 95*, 4784-4788.

Cosio, E. & Bustamante, E. 1984. Subcellular localization of hexokinase in pea leaves. *The Journal of Biological Chemistry 259*, 7688-7692.

Cove, D.J., Kammerer, W., Knight, C.D., Leech, M.J., Martin, C.R. & Wang, T.L. 1991. Developmental genetic studies of the moss, *Physcomitrella patens*. *Symposia of the Society for Experimental Biology 45*, 31-43.

Cove, D.J. & Knight, C.D. 1993. The moss *Physcomitrella patens*, a model system with potential for the study of plant reproduction. *The Plant Cell 5*, 1483-1488.

Dijkwel, P.P, Huijser, C., Weisbeek, P.J., Chua, N.H. & Smeekens, S.C.M. 1997. Sucrose control of phytochrome A signaling in *Arabidopsis*. *The Plant Cell 9*, 583-595.

Entian, K.D. 1980. Genetic and biochemical evidence for hexokinase PII as a key enzyme involved in carbon catabolite repression in yeast. *Molecular and General Genetics 178*, 633-637.

Emanuelsson, O., Nielsen, H., Brunak, S. & von Heijne, G. 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *Journal of Molecular Biology 300*, 1005-1016.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S. & Goodman H.M. 1998. The *Arabidopsis* abscisic acid response locus *ABI4* encodes an Apetala2 domain protein. *The Plant Cell 10*, 1043-1054.

Felgner, P.L., Messer, J.L. & Wilson, J.E. 1979. Purification of a hexokinase-binding protein from the outer mitochondrial membrane. *The Journal of Biological Chemistry 254*, 4946-4949.

Frank, W., Decker, E.L. & Reski, R. 2005. Molecular tools to study *Physcomitrella patens*. *Plant Biology (Stuttgart) 7*, 220-227.

Frommer, W.B. Schulze, W.X. & Lalonde, S. 2003. Hexokinase, jack-of-all-trades. *Science 300*, 261-263.

Galina, A. & da-Silva, W.S. 2000. Hexokinase activity alters sugar-nucleotide formation in maize root homogenates. *Phytochemistry 53*, 29-37.
Gelb, B.D., Adams, V., Jones, S.N., Griffin, L.D., MacGregor, G.R. & McCabe, E.R.B. 1992. Targeting of hexokinase 1 to liver and hepatoma mitochondria. *Proceedings of the National Academy of Sciences of the USA 89*, 202-206.
Gibson, S.I., Laby, R.J & Kim, D. 2001. The *sugar-insensitive1* (*sis1*) mutant of *Arabidopsis* is allelic to *ctr1*. *Biochemical and Biophysical Research Communications 280*, 196-203.
Gibson, S.I. 2004. Sugar and phytohormone response pathways: navigating a signalling network. *Journal of Experimental Botany 55*, 253-264.
Giegé, P., Heazlewood, J.L., Roessner-Tunali, U., Millar, A.H., Fernie, A.R., Leaver, C.J. & Sweetlove, L.J. 2003. Enzymes of glycolysis are functionally associated with the mitochondrion in *Arabidopsis* cells. *The Plant Cell 15*, 2140-2151.
Giese, J., Herbers, K., Hoffmann, M., Klösgen, R. & Sonnewald, U. 2005. Isolation and functional characterization of a novel plastidic hexokinase from *Nicotiana tabacu*m. *FEBS Letters 579*, 827-831.
Goode, J.A., Stead, A.D. & Ducket, J.G. 1992. Redifferentiation of moss protonemata: an experimental and immunofluorescence study of brood cell formation. *Canadian Journal of Botany 71*, 1510-1519.
Graham, I.A., Denby, K.J. & Leaver, C.J. 1994. Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. *The Plant Cell 6*, 761-772.
Halford, N.G., Purcell, P.P. & Hardie, D.G. 1999. Is hexokinase really a sugar sensor in plants? *Trends in Plant Science 4*, 117-120.
Harries, P.A., Pan, A. & Quatrano, R.S. 2005. Actin-related protein 2/3 complex component ARPC1 is required for proper cell morphogenesis and polarized cell growth in *Physcomitrella patens*. *The Plant Cell 17*, 2327-2339.
Heintz, D., Wurtz, V., High, A.A., van Dorsselaer, A., Reski, R. & Sarnighausen, E. 2004. An efficient protocol for the identification of protein phosphorylation in a seedless plant, sensitive enough to detect members of signalling cascades. *Electrophoresis 25*, 1149-1159.
Herrero, P., Martínez-Campa, C. & Moreno, F. 1998. The hexokinase 2 protein participates in regulatory DNA-protein complexes necessary for glucose repression of the *SUC2* gene in *Saccharomyces cerevisiae*. *FEBS Letters 434*, 71-76.
Hiwatashi, Y., Nishiyama, T., Fujita, T. & Hasebe, M. 2001. Establishment of gene-trap and enhancer-trap systems in the moss *Physcomitrella patens*. *The Plant Journal 28*, 105-116.
Hopkins, W. G. 1995. Introduction to plant physiology. John Wiley & Sons, Inc. *The University of Western Ontario, United States of America*.
Horiguchi, G. 2004. RNA silencing in plants: a shortcut to functional analysis. *Differentation 72*, 65-73.
Jang, J.C. & Sheen, J. 1994. Sugar sensing in higher plants. *The Plant Cell 6*, 1665-1679.
Jang, J.C., Léon, P., Zhou, L. & Sheen, J. 1997. Hexokinase as a sugar sensor in higher plants. *The Plant Cell 9*, 5-19.
Johnston, M. 1999. Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. *Trends in Genetics 15*, 29-33.
Keegstra, K. & Cline, K. 1999. Protein import and routing systems of chloroplasts. *The Plant Cell 11*, 557-570.
Knight, C.D., Sehgal, A., Atwal, K., Wallace, J.C., Cove, D.J., Coates, D., Quatrano, R.S., Bahadur, S., Stockley, P.G. & Cuming, A.C. 1995. Molecular responses to abscisic acid and stress are conserved between moss and cereals. *The Plant Cell 7*, 499-506.
Koch, K.E. 1996. Carbohydrate-modulated gene expression in plants. *Annual Review of Plant Physiology and Plant Molecular Biology 47*, 509-540.
Kroemer, K., Reski, R. & Frank, W. 2004. Abiotic stress response in the moss *Physcomitrella patens*: evidence for an evolutionary alteration in signalling pathways in land plants. *Plant Cell Reports 22*, 864-870.
Krysan, P.J., Young, J.C & Sussman, M.R. 1999. T-DNA as an insertional mutagen in *Arabidopsis*. *The Plant Cell 11*, 2283-2290.

Kuser, P.R., Krauchenco, S., Antunes, O.A.C. & Polikarpov, I. 2000. The high resolution crystal structure of yeast hexokinase PII with the correct primary sequence provides new insights into its mechanism of action. *The Journal of Biological Chemistry 275*, 20814-20821.

Laby R.J. Kincaid, M.S., Kim, D. & Gibson, S.I. 2000. The *Arabidopsis* sugar-insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. *The Plant Journal 23*, 587-596.

Lang, D., Eisinger, J., Reski, R. & Rensing, S.A. 2005. Representation and high-quality annotation of the *Physcomitrella patens* transcriptome demonstrates a high proportion of proteins involved in metabolism in mosses. *Plant Biology (Stuttgart) 7*, 238-250.

Lejay, L., Gansel, X., Cerezo, M., Tillard, P., Müller, C., Krapp, A., von Wirén, N., Daniel-Vedele, F. & Gojon, A. 2003. Regulation of root ion transporters by photosynthesis: Functional importance and relation with hexokinase. *The Plant Cell 15*, 2218-2232.

Léon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewsji, N.E., Schwartz, S.H., Zeevaart, J.A. & Koorneef, M. 1996. Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *The Plant Journal 10*, 655-661.

Lindén, M., Gellerfors, P. & Nelson, B.D. 1982. Pore protein and the hexokinase-binding protein from the outer membrane of rat liver mitochondria are identical. *FEBS Letters 141*, 189-192.

Maitra, P.K. & Lobo, Z. 1983. Genetics of yeast glucokinase. *Genetics 105*, 501-515.

Martienssen, R.A. 1998. Functional genomics: Probing plant gene function and expression with transposons. *Proceedings of the National Academy of Sciences of the USA 95*, 2021-2026.

Martin, T., Hellman, H., Schnidt, R., Willmitzer, L. & Frommer, W.B. 1997. Identification of mutants in metabolically regulated gene expression. *The Plant Journal 11*, 53-62.

Mengiste, T. & Paszkowski. 1999. Prospects for the precise engineering of plant genomes by homologous recombination. *Biological Chemistry 380*, 749-758.

Miernyk, J.A. & Dennis, D.T. 1983. Mitochondrial, plastid and cytosolic isozymes of hexokinase from developing endosperm of *Ricinus communis*. *Archives of Biochemistry and Biophysics 226*, 458-468.

Mita, S., Murano, N., Akaike, M. & Nakamura, K. 1997a. Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for β-amylase and on the accumulation of anthocyanin that are inducible by sugars. *The Plant Journal 11*, 841-851.

Mita, S., Hirano, H. & Nakamura, K. 1997b. Negative regulation in the expression of a sugar-inducible gene in *Arabidopsis thaliana*: A recessive mutation causing enhanced expression of gene for β-amylase. *Plant Physiology 114*, 575-582.

Moore, B.D. & Sheen, J. 1999. Plant sugar sensing and signalling-a complex reality. *Trends in Plant Science 4*, 250.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Hwang, I., Jones, T. & Sheen, J. 2003. Role of the *Arabidopsis* glucose sensor *HXK1* in nutrient, light, and hormone signaling. *Science 300*, 332-336.

Moreno, F., Ahuatzi, D., Riera, A., Palomino, C.A. & Herrero, P. 2005. Glucose sensing through the Hxk2-dependent signalling pathway. *Biochemical Society Transactions 33*, 265-268.

Murata, T., Katagiri, H., Ishihara, H., Shibasaki, Y., Asano, T., Toyoda, Y., Pekiner, B., Pekiner, C., Miwa, I. & Oka, Y. 1997. Co-localization of glucokinase with actin filaments. *FEBS Letters 406*, 109-113.

Nagao, M., Minami, A., Arakawa, K., Fujikawa, S. & Takezawa, D. 2005. Rapid degradation of starch in chloroplasts and concomitant accumulation of soluble sugars associated with ABA-induced freezing tolerance in the moss *Physcomitrella patens*. *Journal of Plant Physiology 162*, 169-180

Németh, K., Salchert, K., Putnoky, P., Bhalerao, R., Koncz-Kálmán, Z., Stankovic-Stangeland, B., Bakó, L., Mathur, J., Ökrész, L., Stabel, S., Geigenberger, P., Stitt, M., Rédei, G.P., Schell, J. & Koncz, C. 1998. Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein in *Arabidopsis*. *Genes and Development 12*, 3059-3073.

Neuhaus, H.E. & Emes, M.J. 2000. Nonphotosynthetic metabolism in plastids. *Annual Review of Plant Physiology and Plant Molecular Biology 51*, 111-140.
Nishiyama, T., Hiwatashi, Y., Sakakibara, K., Kato, M. & Hasebe, M. 2000. Tagged mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle mutagenesis. *DNA Research 7*, 9-17.
Nishiyama, T., Fujita, T., Shin-I, T., Seki, M., Nishide, H., Uchiyama, I., Kamiya, A., Carninci, P., Hayashizaki, K., Kohara, Y. & Hasebe, M. 2003. Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: Implication for land plant evolution. *Proceedings of the National Academy of Sciences of the USA. 100*, 8007-8012.
Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering 10*, 1-6.
Olsson, T., Thelander, M. & Ronne, H. 2003. A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. *The Journal of Biological Chemistry 278*, 44439-44447.
Plaxton, W.C. 1996. The organization and regulation of plant glycolysis. *Annual Review of Plant Physiology and Plant Molecular Biology 47*, 185-214.
Pego, J.V., Kortstee, A.J., Huijser, C. & Smeekens, S.C.M. 2000. Photosynthesis, sugars and the regulation of gene expression. *Journal of Experimental Botany 51*, 407-416.
Pego, J.V. & Smeekens, S.C.M. 2000. Plant fructokinases: a sweet family get-together. *Trends in Plant Science 5*, 531-536.
Preller, A. & Wilson, J.E. 1992. Localization of the type III isozyme of hexokinase at the nuclear periphery. *Archives of Biochemistry and Biophysics 294*, 482-492.
Randez-Gil, F., Sanz, P., Entian, K.D. & Prieto, J. 1998a. Carbon source-dependent phosphorylation of hexokinase PII and its role in the glucose-signaling response in yeast. *Molecular and Cellular Biology 18*, 2940-2948.
Randez-Gil, F., Herrero, P., Sanz, P., Prieto, J.A. & Moreno, F. 1998b. Hexokinase PII has a double cytosolic-nuclear localisation in *Saccharomyces cerevisiae*. *FEBS Letters 425*, 475-478.
Rensing, S.A., Rombauts, S., Van de Peer, Y. & Reski, R. 2002. Moss transcriptome and beyond. *Trends in Plant Science 7*, 535-538.
Rensing, S.A., Kiessling, J., Reski, R. & Decker, E.L. 2004. Diversification of *ftsZ* during early land plant evolution. *Journal of Molecular Evolution 58*, 154-162.
Rensing, S.A., Fritzowsky, D., Lang, D. & Reski, R. 2005. Protein encoding genes in an ancient plant: analysis of codon usage, retained genes and splice sites in a moss, *Physcomitrella patens*. *BMC Genomics 6*, 43.
Reski, R. & Abel, W.O. 1985. Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. *Planta 165*, 354-358.
Reski, R., Faust, M., Wang, X.-H., Wehe, M. & Abel, W.O. 1994. Genome analysis of the moss *Physcomitrella patens (Hedw.) B.S.G. Molecular and General Genetics 244*, 352-359.
Reski, R. 1998. Development, genetics and molecular biology of mosses. *Botanical Acta 111*, 1-15.
Reski, R. 2005. Do we need another model plant? *Plant Biology (Stuttgart) 7*, 219.
Rolland, F. Moore, B. & Sheen, J. 2002. Sugar sensing and signaling in plants. *The Plant Cell 14*, S185-S205.
Rolland, F., Winderickx, J. & Thevelein, J.M. 2002. Glucose-sensing and -signalling mechanisms in yeast. *FEMS Yeast Research 2*, 183-201.
Ronne, H. 1995. Glucose repression in fungi. *Trends in Genetics 11*, 12-17.
Rook, F., Corke, F., Card, R., Munz, G., Smith, C. & Bevan, M.W. 2001. Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. *The Plant Journal 26*, 421-433.
Schaefer, D., Zrÿd, J.P., Knight, C.D. & Cove, D.J. 1991. Stable transformation of the moss *Physcomitrella patens*. *Molecular and General Genetics 226*, 418-424.
Schaefer, D.G. & Zrÿd, J.-P. 1997. Efficient gene targeting in the moss *Physcomitrella patens*. *The Plant Journal 11*, 1195-1206.

Schaefer, D.G. 2001. Gene targeting in *Physcomitrella patens*. *Current Opinion in Plant Biology 4*, 143-150.
Schaefer, D.G. 2002. A new moss genetics: Targeted mutagenesis in *Physcomitrella patens*. *Annual Review of Plant Biology 53*, 477-501.
Schnarrenberger, C. 1990. Characterization and compartmentation, in green leaves, of hexokinases with different specificities for glucose, fructose, and mannose and for nucleoside triphosphates. *Planta 181*, 249-255.
Schulte, J. & Reski, R. 2004. High throughput cryopreservation of 140 000 *Physcomitrella patens* mutants. *Plant Biology (Stuttgart) 6*, 119-127.
von Schwartzenberg, K., Kruse, S., Reski, R., Moffatt, B. & Laloue, M. 1998. Cloning and characterization of an adenosine kinase from *Physcomitrella* involved in cytokinin metabolism. *The Plant Journal 13*, 249-257.
Schween, G., Gorr, G., Hohe, A. & Reski, R. 2003. Unique tissue-specific cell cycle in *Physcomitrella patens*. *Plant Biology (Stuttgart) 5*, 1-9.
da-Silva, W.S., Rezende, G.L. & Galina, A. 2001. Subcellular distribution and kinetic properties of cytosolic and non-cytosolic hexokinases in maize seedling roots: implications for hexose phosphorylation. *Journal of Experimental Botany 52*, 1191-1201.
Singh, K.K., Chen, C. Epstein, D.K. & Gibbs, M. 1993. Respiration of sugars in spinach (*Spinacia oleracea*), maize (*Zea mays*), and *Chlamydomonas reinhardtii* F-60 chloroplasts with emphasis on the hexose kinases. *Plant Physiology 102*, 587-593.
Struhl, K. 1983. The new yeast genetics. *Nature 5*, 391-397.
Sui, D. & Wilson, J.E. 1997. Structural determinants for the intracellular localization of the isozymes of mammalian hexokinase: intracellular localization of fusion constructs incorporating structural elements from hexokinase isozymes and the green fluorescent protein. *Archives of Biochemistry and Biophysics 345*, 111-125.
Tanner, G.J., Copeland, L. & Turner, J.F. 1983. Subcellular localization of hexose kinases in pea stems: mitochondrial hexokinase. *Plant Physiology 72*, 659-663.
Thelander, M., Olsson, T. & Ronne, H. 2004. Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle. *The EMBO Journal 23*, 1900-1910.
Theissen, G., Münster, T. & Henschel, K. 2001. Why don't mosses flower? *New Phytologist 150*, 1-8.
Tiessen, A., Prescha, K., Branscheid, A., Palacious, N., McKibbin R., Halford, N.G. & Geigenberger, P. 2003. Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *The Plant Journal 35*, 490-500.
Tucker, E.B., Lee, M., Alli, S., Sookhdeo, V., Wada, M., Imaizumi, T., Kasahara, M. & Hepler, P.K. 2005. UV-A induces two calcium waves in *Physcomitrella patens*. *Plant and Cell Physiology 46*, 1226-1236.
Uenaka, H., Wada M. & Kadota, A. 2005. Four distinct photoreceptors contributes to light-induced side branch formation in the moss *Physcomitrella patens*. *Planta 21*, 1-9.
Weber, A., Servaites, J.C., Geiger, D.R., Kofler, H., Hille, D., Gröner, F., Hebbeker, U. Flügge, U.-I. 2000. Identification, purification and molecular cloning of a putative plastidic glucose translocator. *The Plant Cell 12*, 787-801.
Veramendi, J., Roessner, U., Renz, A., Willmitzer, L. & Trethewey, R. N. 1999. Antisense repression of hexokinase 1 leads to an overaccumulation of starch in leaves of transgenic potato plants but not to significant changes in tuber carbohydrate metabolism. *Plant Physiology 121*, 123-133.
Veramendi, J., Fernie, A.R., Leisse, A., Willmitzer, L. & Trethewey, R.N. 2002. Potato hexokinase 2 complements transgenic *Arabidopsis* plants deficient in hexokinase 1 but does not play a key role in tuber carbohydrate metabolism. *Plant Molecular Biology 49*, 491-501.
Wiese, A., Gröner, F., Sonnewald, U., Deppner, H., Lerchl, J., Hebbeker, U., Flügge, U. & Weber, A. 1999. Spinach hexokinase I is located in the outer envelope membrane of plastids. *FEBS Letters 461*, 13-18.
Wilson, J.E. 2003. Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function. *The Journal of Experimental Biology 206*, 2049-2057.

Xiao, W., Sheen, J. & Jang, J.C. 2000. The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Molecular Biology 44*, 451-461.

Xie, G.C. & Wilson, J.E. 1988. Rat brain hexokinase: the hydrophobic N-terminus of the mitochondrially bound enzyme is inserted in the lipid bilayer. *Archives of Biochemistry and Biophysics 267*, 803-810.

Yanagisawa, S., Yoo, S.D. & Sheen, J. 2003. Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. *Nature 425*, 521-525.

Zhou, L., Jang, J.C., Jones, T.L. & Sheen, J. 1998. Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proceedings of the National Academy of Sciences of the USA 95*, 10294-10299.

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