Studies of Molecular Mechanisms Integrating Carbon Metabolism and Growth in Plants

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Abstract

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Plants use light energy, carbon dioxide and water to produce sugars and other carbohydrates, which serve as stored energy reserves and as building blocks for biosynthetic reactions. Supply of light is variable and plants have evolved means to adjust their growth and development accordingly. An increasing body of evidence suggests that the basic mechanisms for sensing and signaling energy availability in eukaryotes are evolutionary conserved and thus shared between plants, animals and fungi.

I have used different experimental approaches that take advantage of findings from other eukaryotes in studying carbon and energy metabolism in plants. In the first part, I developed a novel screening procedure in yeast aimed at isolating cDNAs from other organisms encoding proteins with a possible function in sugar sensing or signaling. The feasibility of the method was confirmed by the cloning of a cDNA from *Arabidopsis thaliana* encoding a new F-box protein named AtGrh1, which is related to the yeast Grr1 protein that is involved in glucose repression.

In the second part of the study, plant homologues of key components in the yeast glucose repression pathway were cloned and characterized in the moss *Physcomitrella patens*, in which gene function can be studied by gene targeting. We first cloned *PpHXK1* which was shown to encode a chloroplast localized hexokinase representing a previously overlooked class of plant hexokinases with an N-terminal chloroplast transit peptide. Significantly, PpHxk1 is the major hexokinase in *Physcomitrella*, accounting for 80% of the glucose phosphorylating activity. A knockout mutant deleted for *PpHXK1* exhibits a complex phenotype affecting growth, development and sensitivities to plant hormones.

I also cloned and characterized two closely related *Physcomitrella* genes, *PpSNF1a* and *PpSNF1b*, encoding type 1 Snf1-related kinases. A double knockout mutant for these genes was viable even though it lacks detectable Snf1-like kinase activity. The mutant suffers from pleiotropic phenotypes which may reflect a constitutive high energy growth mode. Significantly, the double mutant requires constant high light and is therefore unable to grow in a normal day/night light cycle. These findings are consistent with the proposed role of the Snf1-related kinases as energy gauges which are needed to recognize and respond to low energy conditions.

Keywords: AMPK, hexokinase, glucose repression, Grr1, Snf1, SnRK1

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Appendix

Papers I-IV

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

I. Thelander, M., Fredriksson, D., Schouten, J., Hoge, H. & Ronne, H. 2002. Cloning by pathway activation in yeast: identification of an *Arabidopsis thaliana* F-box protein that turns on glucose repression. *Plant Mol. Biol. 49*, 67-79.

II. 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*. *J. Biol. Chem. 278*, 44439-44447.

III. Thelander, M., Olsson, T. & Ronne, H. 2003. Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle. (Manuscript)

IV. Thelander, M., Olsson, T. & Ronne, H. 2003. Phenotypic characterization of protonemal growth and development in a *Physcomitrella patens* hexokinase knockout mutant. (Manuscript)

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Abbreviations

ABA	abscisic acid
AMP	adenosine monophosphate
ATP	adenosine triphosphate
cDNA	complementary DNA
DNA	deoxyribonucleic acid
EST	expressed sequence tag
GFP	green fluorescent protein
SCF	Skp1, Cullin, F-box (-complex)
LRR	leucine rich repeats
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase PCR
YTH	yeast two hybrid
6-dGlc	6-deoxy-D-glucose
3-O-mGlc	3-ortho-methylglucose

Introduction

Energy, carbon and life

Plants use light energy, carbon dioxide and water to produce sugars and other carbohydrates with energy stored in their chemical bonds. These carbohydrates can either be used directly or stored as energy reserves for future use both by the photosynthetic cell and by other tissues within the plant. Plant growth and development is in the long run dependent on a sufficient energy supply in the form of light. The supply of light is, however, highly variable and plants must therefore be able to monitor changes in the current energy status of cells and tissues, and respond to such changes by either accumulating or mobilizing storage compounds. This is in part achieved by monitoring carbohydrate levels as a measure of stored and allocated energy. The mechanisms involved are complex and their details remain to be elucidated, but it is clear that this metabolic regulation to some extent is mediated by evolutionary conserved proteins that are present also in other eukaryotes.

The eukaryotic organism in which mechanisms for monitoring and responding to sugar levels have been studied in most detail is the yeast *Saccharomyces cerevisiae*. It has a versatile metabolic machinery which is largely regulated by sugar levels. Yeast normally grows in habitats, such as on the skin of grapes, where it is surrounded by other microorganisms. These organisms all compete for limited and fluctuating amounts of carbon compounds such as sugars for their energy supply. Efficient utilization of sugars and rapid adaptation to sudden changes in their abundance is therefore a prerequisite for survival. For this reason, yeast has multiple systems that can monitor the amounts of both intracellular and extracellular sugars. These systems regulate the expression and activities of enzymes and transporters that in turn ensure a rapid and efficient utilization of available carbon sources.

It should be clearly stated that plant cells differ fundamentally from yeast cells in that they are photosynthetic and have to integrate their metabolic activities with other cells and tissues within the same plant. Still, both yeast and plant cells have systems that monitor sugar levels as a measure of energy availability. An increasing body of evidence suggests that there are evolutionary conserved mechanisms involved, and that one can learn things about how plant cells monitor and respond to energy availability from other eukaryotes such as yeast.

Carbon metabolism in yeast

Metabolic pathways

Biological research today is largely focused on model organisms that are chosen due to their usefulness in experimental biology. One such organism is the unicellular budding yeast *Saccharomyces cerevisae* which has served as a model in studies of many fundamental eukaryotic cellular processes (Broach, Pringle & Jones, 1991). For convenience, *S. cerevisiae* will simply be referred to as yeast in the text below.

Yeast cells can utilize a number of different carbon compounds for their energy supply but they prefer to ferment glucose. During such preferred fermentative growth, energy is released by glycolytic degradation of glucose into ethanol. Respiratory functions are therefore dispensable in the presence of glucose. The preference for fermentative growth is believed to give yeast an advantage in the competition with other micro-organisms (Rolland, Winderickx & Thevelein, 2002). Unlike many competitors, yeast has the ability to adapt its metabolism for use of other carbon sources, including oxidative metabolism of ethanol, once the glucose has been consumed. Furthermore, yeast cells can stand high ethanol concentrations in their surroundings while the growth of many competing micro-organisms is inhibited by ethanol.

Yeast cells exclusively ferment glucose when it is available. Other carbon sources are left non-utilized until the glucose has been consumed. This is achieved by a combination of two principally different mechanisms; glucose induction (Figure 1) and glucose repression (Figure 3). Functions that are needed for rapid uptake and metabolism of glucose are stimulated by glucose. In contrast, functions which are dispensable for the fermentation of glucose are blocked in the presence of glucose by transcriptional repression. In addition to the glucose induction and repression pathways, yeast cells also have additional systems to monitor glucose availability, such as the RAS-cAMP pathway (see Rolland, Winderickx & Thevelein, 2002 and references therein).

The yeast glucose induction pathway

Functions that are stimulated by glucose include glucose transport and the activation of glycolytic enzymes. The latter is achieved by allosteric regulation of enzymes as well as an up-regulation of the corresponding genes. The actual trigger appears to be an increase in glycolytic intermediates rather than glucose itself (see Rolland, Winderickx & Thevelein, 2002 and references therein). In contrast, the stimulation of glucose transport is mediated by the main glucose induction pathway in which glucose is the molecule being sensed (Figure 1).

There are 17 hexose transporter (*HXT1-HXT17*) genes in the yeast genome and at least seven of them encode functional proteins (reviewed by Özcan & Johnston, 1999). Some *HXT* genes encode high affinity transporters with low capacity while others encode low affinity transporters with high capacity. The transcription of these genes is glucose regulated in a way that ensures that an optimal set of transporters is expressed at any time. Thus, the transcription of all *HXT* genes is repressed in the absence of glucose. This repression is relieved by low glucose concentrations through the action of the main glucose induction pathway (Figure 1). The picture is further complicated by additional layers of regulation (Özcan & Johnston, 1995). For example, some high affinity transporters are also repressed by high levels of glucose to ensure that their expression is limited to conditions of low glucose availability. This additional regulation is achieved by the major glucose repression pathway which is described below. Furthermore, there also exists an uncharacterized mechanism which ensures that the major low affinity transporter is exclusively expressed in the presence of high glucose concentrations.

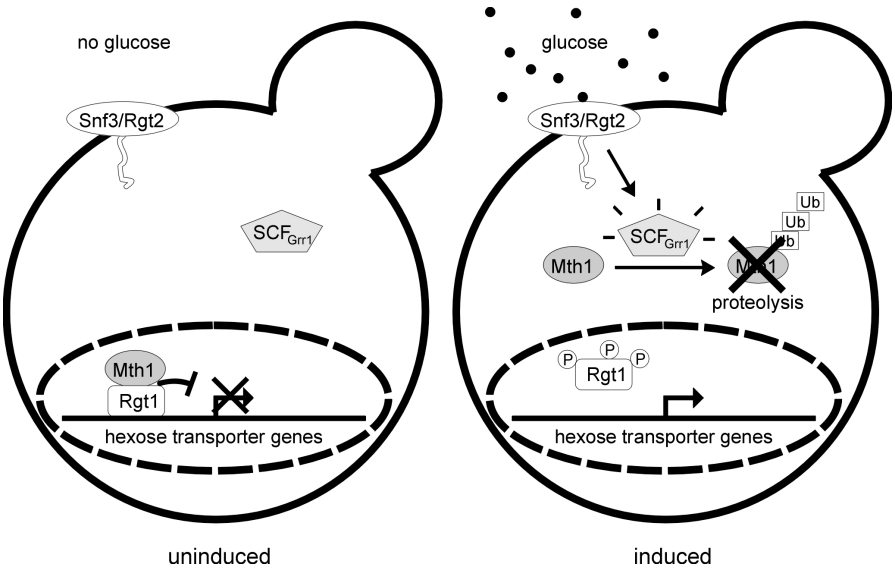

Figure 1. Schematic overview of the yeast glucose induction pathway.

Transcriptional repression of the *HXT* genes in the absence of glucose is mediated by the zinc-finger-containing DNA binding repressor Rgt1 (Marshall-Carlson *et al*., 1991). The signal triggering derepression is believed to be generated by the extracellular interaction of glucose with two homologous sensors called Snf3 (Neigeborn & Carlson, 1984) and Rgt2 (Marshall-Carlson *et al*., 1991). Both these membrane spanning proteins are related to the hexose transporters (Hxt1-Hxt17) but possess extended cytoplasmic tails (Özcan *et al*., 1996). Snf3 and Rgt2 appear to have lost their ability to transport sugars and instead gained importance as sensors needed for glucose relaxation of *HXT* gene repression (Özcan, Dover & Johnston, 1998). Glucose triggered derepression of the *HXT* genes has also been shown to require a functional copy of the *GRR1* gene (Flick & Johnston, 1991). This requirement can be suppressed by mutating the repressor Rgt1, and Grr1 has therefore been suggested to act upstream of Rgt1 in the pathway (Özcan & Johnston, 1995).

The *GRR1* gene encodes a large 1151 amino acid residue protein with an N-terminal F-box motif followed by a number of leucine-rich repeats (LRRs) (Flick & Johnston, 1991). F-box proteins from animals, plants and fungi are known to be part of protein complexes which serve as E3 ubiquitin ligases (Patton, Willems & Tyers, 1998; Risseeuw *et al*., 2003). These complexes are referred to as SCF complexes which comes from the names of their three evolutionary conserved components; Skp1, Cullin and F-box proteins. E3 ubiquitin ligases act together with E1 ubiquitin activating enzymes and E2 ubiquitin conjugating enzymes to tag proteins for degradation by polyubiquitinylation (Figure 2). F-box proteins are believed to act as the specificity determinants in these SCF complexes. Thus, they bind substrates through protein-protein interaction domains such as the LRRs in

the case of Grr1. The F-box of Grr1 and related proteins mediates interaction with the SCF complex by binding to Skp1 (Li & Johnston, 1997).

It should be emphasized that Grr1 has been implicated also in processes which are unrelated to the glucose induction pathway (see Li & Johnston, 1997 and references therein). It was initially proposed that the target of Grr1 mediated proteolysis in the glucose induction pathway could be the actual repressor Rgt1 (Li & Johnston, 1997). However, a recent report by Flick *et al*. (2003) suggests that the target of Grr1 action is a protein called Mth1 (Hubbard, Jang & Carlson, 1994) which, together with a related protein called Std1 (Hubbard, Jang & Carlson, 1994) has been connected to the glucose induction pathway by genetic interactions (Schmidt *et al*., 1999; Schulte *et al*., 2000). Mth1 and Std1 have also been shown to interact physically with the cytoplasmic tails of the glucose sensors Snf3 and Rgt2 (Schmidt *et al*., 1999). Flick *et al*. (2003) showed that the activity of Rgt1 is regulated by phosphorylation and that hyperphosphorylation results in dissociation from the target promoters and derepression. In addition, Mth1 is needed to suppress Rgt1 hyperphosphorylation and as a consequence, to maintain repression. Finally, evidence was provided that support the notion that Mth1 is the actual target of glucose regulated Grr1 dependent proteolysis.

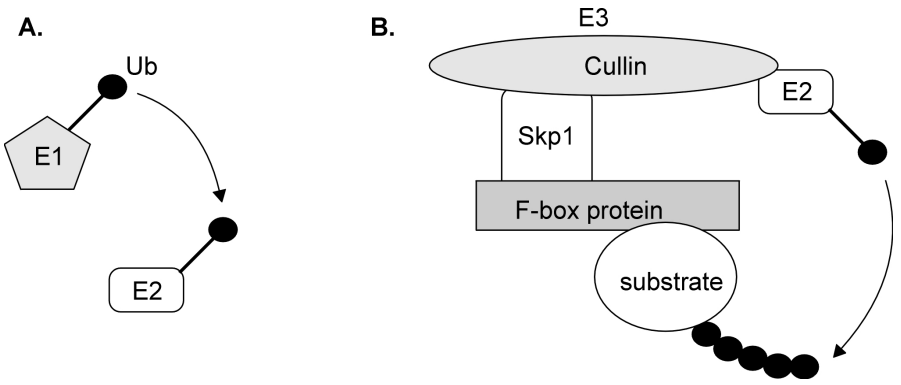

Figure 2. Overview of SCF-mediated polyubiquitinylation of protein substrates leading to their subsequent degradation (Itoh, Matsuoka & Steber, 2003).

The yeast glucose repression pathway

A variety of yeast genes encoding functions that are dispensable during fermentative growth are transcriptionally repressed in the presence of glucose (Figure 3) (Ronne 1995; Johnston 1999; Rolland, Winderickx & Thevelein, 2002). Examples of such functions are respiration, gluconeogenesis and the uptake and metabolism of alternative carbon sources. This repression is maintained by the Mig1 zinc finger protein (Nehlin & Ronne, 1990) that binds to sequences in the

promoters of target genes (Nehlin & Ronne, 1990; Nehlin, Carlberg & Ronne, 1991) and recruits the general co-repressor complex Ssn6/Tup1 (Keleher, 1992; Treitel & Carlson, 1995). The Mig1 repressor is regulated by glucose dependent phosphorylation resulting in a differential nucleo-cytoplasmic localization (De Vit, Waddle & Johnston, 1997). Thus, the Mig1 repressor is phosphorylated in the absence of glucose resulting in its rapid translocation to the cytoplasm. This will relieve repression of the target genes, and therefore allow expression of functions needed for metabolism of alternative carbon sources. A second zinc finger protein, Mig2, is partially redundant with Mig1 in the repression of some genes, such as the *SUC2* gene encoding invertase (Lutfiyya & Johnston, 1996).

The protein which is responsible for phosphorylation of Mig1 is a protein kinase called Snf1 (Östling & Ronne, 1998; Treitel, Kuchin & Carlson, 1998). The activity of the Snf1 kinase is negatively regulated by glucose (Woods *et al*., 1994). The name *SNF1* (Sucrose Non Fermenting 1) comes from the fact that the *snf1* mutant fails to grow on sucrose (Carlson, Osmond & Botstein, 1981). The *SNF1* gene is in fact essential for growth on any carbon source other than glucose since it is needed for the derepression of glucose repressed genes (Celenza & Carlson, 1984). Uptake of glucose into the cell is required but not sufficient to generate the glucose repression signal that inhibits Snf1 activity (Reifenberger, Boles & Ciriacy, 1997; Ye *et al*, 1999). This suggests that glucose is sensed intracellularly by a mechanism that differs from the one triggering the glucose induction pathway.

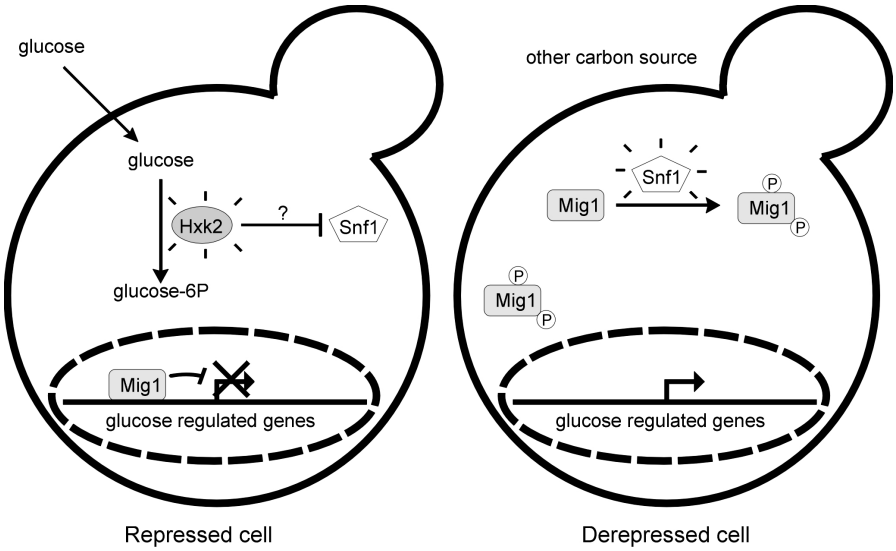

Figure 3. Schematic overview of the yeast glucose repression pathway.

The first enzymatic reaction in the fermentation of glucose is hexokinase mediated phosphorylation of the sugar. There are three hexose phosphorylating enzymes in yeast, one of which is encoded by the *HXK2* gene (Frohlich, Entian &

Mecke, 1985). The Hxk2 gene product is the major hexokinase in yeast, and differs from the other two since it does not only provide a metabolic function but is also essential for glucose repression (Entian, 1980). Thus, Hxk2 is needed for glucose sensing but the exact mechanism remains elusive (see Rolland, Winderickx & Thevelein, 2002 and references therein). It could act as a true intracellular glucose sensor and generate a signal upon glucose interaction that affects downstream components such as the Snf1 kinase. Such a sensing function may be dependent or independent on its catalytic activity. Alternatively, Hxk2 may simply provide glucose phosphorylating capacity and one of several possible downstream products may then trigger the actual signaling. Support for this notion comes from the fact that other hexokinases can in part complement the signaling function of Hxk2 when overexpressed (Rose, Albig & Entian, 1991).

Carbon metabolism in plants

Metabolic pathways

Plants are photoautotrophic which means that they utilize inorganic compounds and light for all biosynthesis (Buchanan, Gruissem & Jones, 2000). Unlike animals and fungi, plants therefore need a complete set of pathways for synthesis and metabolism of all essential carbon compounds. Photosynthesis generates ATP and reducing power used to produce triose phosphates. Triose phosphates are simple carbohydrates that can be used as substrates in multiple anabolic and catabolic processes. They can be converted to hexose phosphates which are used for hexose, sucrose, starch and cell wall synthesis. Via the penthose phosphate pathway they can also be used to generate reducing power for biosynthesis of fatty acids as well as building blocks for nucleic acid and amino acid synthesis. Furthermore, they can be regarded as the principal substrates for glycolysis and downstream respiration. The latter two processes are primarily energy releasing catabolic pathways but they also supply anabolic pathways with organic building blocks. Photosynthesis occurs in the chloroplasts. These organelles belong to a family of plant specific compartments called plastids, which are separated from the cytoplasm by a double envelope that blocks diffusion of biomolecules. A significant proportion of plant biosynthesis is performed within the plastids. Some enzymatic reactions within the carbohydrate metabolic network briefly described above occur in plastids while others occur in the cytoplasm. Controlled transport of substrates, cofactors and products in and out of the plastids is therefore of key importance to sustain a functional metabolic network in plant cells.

Carbon allocation and storage

Their dependence on photosynthesis makes plants potentially vulnerable to light fluctuations such as the natural day-night cycle. Light exposure also varies with the position of individual tissues within a plant, ranging from exposed leaves to roots below the ground. These light variations cause *de novo* synthesis of triose phosphates through photosynthesis to vary dramatically between different cells and tissues within a plant. To cope with these variations higher plants have evolved means to transport and store energy as carbohydrates. Plant parts that need more energy than they can generate themselves are called sink tissues. Sink

tissues are fed by photosynthetically active source tissues that generate more energy than they need. The most common way to move energy from source to sink tissues in higher plants is vascular transport of sucrose (Lalonde *et al*., 1999).

Sucrose is synthesized in the cytoplasm of source tissues from UDP-glucose and fructose-6-P by the enzymes sucrose phosphate synthase and sucrose phosphate phosphorylase (Fernie, Willmitzer & Trethewey, 2002). It is then transported to sink tissues (Lalonde *et al*., 1999) where it is cleaved into glucose and fructose by invertase or alternatively, into UDP-glucose and fructose by sucrose synthase (Fernie, Willmitzer & Trethewey, 2002). The imported hexoses are either used directly by sink cells to meet energy and biosynthetic demands, or alternatively used to synthesize starch in the plastids for storage. Excess photosynthesis in source tissues during the day can make sucrose synthesis and/or transport rate limiting factors. In such cases, excess carbohydrates are stored as transitory starch in the chloroplasts of the source cells. This starch is then mobilized during the night by phosphorylytic or hydrolytic starch degradation. The excess of glucose-1-P or glucose which is not needed by the source cell is converted into sucrose and transported to sink tissues.

Sugars as signaling molecules in plants

Sugars are known to affect plant growth and development in many ways and it is clear that they do so in two fundamentally different ways. First, sugars are important as substrates in carbon and energy metabolism in both source and sink tissues and therefore naturally affect their growth. Second, an increasing body of evidence suggests that sugars also have a function as hormone-like signaling molecules (Rolland, Moore & Sheen, 2002). Sugar abundance is a good measure of the current energy status of cells and tissues. Accordingly, it is not surprising that also plants have systems, similar to those already described for yeast, to directly monitor and respond to changes in sugar abundance. Given that sugars in higher plants are transported between cells and tissues, these systems are also believed to have a function in long range coordination of energy homeostasis and metabolism. The signaling effects of sugars are believed to cause changes in gene expression which in turn affect metabolism, growth and development. A large number of sugar responsive genes have been described and their functions suggest that sugars regulate a wide range of metabolic processes. Thus, low levels of sugars appear to enhance photosynthesis and the mobilization of energy reserves, while high sugar levels promote growth and carbohydrate storage (Koch, 1996). In addition to the regulation of gene expression, plant sugar signaling has also been proposed to directly regulate the activity of certain key metabolic enzymes (Sugden *et al*., 1999; Tiessen *et al*., 2003).

Cross-talk between sugar signaling and phytohormone signaling

Plants are complex biological systems in which cells and tissues with quite different morphology and function are dependent on each other for survival. Growth and development are dictated by a combination of genetic programs and environmental inputs. The coordination of such regulation between cells and tissues is largely mediated by phytohormones (Buchanan, Gruissem & Jones,

2000). Among others, these include abscisic acid (ABA), ethylene, auxins and cytokinins.

As already stated, also sugars such as glucose can act as hormone-like signals to regulate gene expression, growth and development in plants. Glucose has for example been shown to inhibit early seedling development and photosynthetic gene expression in *Arabidopsis thaliana*. These findings have been used by several independent research groups as the bases for genetic screens aimed at isolating sugar-insensitive or sugar-hypersensitive mutants (Pego, Weisbeek & Smeekens, 1999; Dijkwel *et al*., 1997; Martin *et al*., 1997; Laby *et al*., 2000; Zhou *et al*., 1998; Mita *et al*., 1997; Mita, Hirano & Nakamura, 1997). Interestingly, several mutants isolated in this way proved to be allelic to previously described mutants implicated in phytohormone synthesis or signaling. Thus, the sugar insensitive *gin1/sis4/isi4* and *gin6/sun6/sis5/isi3* mutants are allelic to the ABA biosynthesis mutant *aba2* and the ABA insensitive mutant *abi4*, respectively (Laby *et al*., 2000; Rook *et al*., 2001; Arenas-Huertero, 2000; Huijser *et al*., 2000). Although a detailed molecular explanation is pending, these observations suggest a link between sugar signaling and ABA signaling (Rolland, Moore & Sheen, 2002 and references therein).

Furthermore, the sugar insensitive *gin4/sis1* mutant has been shown to be allelic to the constitutive ethylene signaling mutant *ctr1*, suggesting that a link exists also between sugar signaling and ethylene signaling (Gibson, Laby & Kim, 2001). This notion is further supported by the observation that the constitutive ethylene biosynthesis mutant *eto1* is insensitive to glucose while the ethylene insensitive *etr1*, *ein2* and *ein3* mutants are glucose hypersensitive (Rolland, Moore & Sheen, 2002). The stability of the Ein3 transcription factor, which mediates ethylene induction of gene expression, was recently shown to be enhanced by ethylene while it is negatively regulated by glucose (Yanagisawa, Yoo & Sheen, 2003). Thus, the crosstalk between sugar and ethylene signaling is at least partially explained by hexokinase mediated (see below) glucose regulated degradation of Ein3.

In addition, phenotypic characterization of the glucose insensitive mutant *gin2*, shown to encode a catalytically inactive hexokinase 1, has also suggested a link between sugar signaling and auxin/cytokinin signaling (Moore *et al*., 2003). Thus, the *gin2* mutant exhibits a defect in auxin-induced cell proliferation and root formation while it is hypersensitive to cytokinin promoted shoot induction. Furthermore, constitutive cytokinin signaling mutants as well as auxin signaling-deficient mutants are resistant to glucose repression of seedling development (Moore *et al*., 2003). Although the details remain elusive, these observations further emphasize the connection between auxin/cytokinin signaling and glucose signaling.

Sugar sensing and signaling in plants

Classification of sugar sensing mechanisms in plants

There exist multiple systems for monitoring sugar levels in plants and different authors have suggested that several parallel signaling pathways are involved

(Smeekens & Rook, 1997; Koch *et al*., 2000; Xiao, Sheen & Jang, 2000; Rolland, Moore & Sheen, 2002). These have been classified according to the different sugars or sugar derivates that are believed to trigger signaling in each pathway. An inherent complication is the fact that many of the molecules of interest are more or less inter-convertible. Several different sugar sensing and signaling pathways may coexist and their relative importance may vary with species, tissue type and growth conditions.

A sucrose-specific sensing system has been proposed to exist, which would be independent of further metabolism of this sugar (Chiou & Bush, 1998; Rook *et al*., 1998; Tiessen *et al*., 2003). This has been suggested because the products of sucrose hydrolysis, glucose and fructose, are less potent triggers than sucrose itself. The exact mechanism of sucrose sensing is yet to be elucidated, but Lalonde *et al*. (1999) have proposed that a sucrose sensor is present in the plasma membrane. The existence of such a sensor is supported by the finding that disaccharide analogues which cannot be further metabolized are able to mimic sucrose in triggering regulation of gene expression and enzyme activity (Loreti, Alpi & Perata, 2000; Fernie, Roessner & Geigenberger, 2001). In this context, it should also be mentioned that the disaccharide trehalose has been implicated as a trigger of a signal pathway that regulates gene expression, metabolism and development in plants (Eastmond and Graham, 2003).

In addition to disaccharide sensing, there are also mechanisms that monitor the abundance of glucose and other hexoses. Different glucose sensing mechanisms have been classified based on their dependence on the hexose phosphorylating enzyme hexokinase (Xiao, Sheen & Jang, 2000). Hexokinase independent glucose regulation of gene expression has been demonstrated for genes encoding apoplastic invertase in *C. rubrum* as well as ADP-glucose pyrophosphorylase, chalcone synthase, phenylalanine ammonia-lyase, and asparagine synthetase in *Arabidopsis* (Roitsch, Bittner & Godt, 1995; Xiao, Sheen & Jang, 2000). Hexokinase independent signaling is unaffected by transgenic manipulation of hexokinase activity (Xiao, Sheen & Jang, 2000) and it can be triggered by glucose analogues (6-dGlc and 3-O-mGlc) which are not substrates for hexokinase (Roitsch, Bittner & Godt, 1995; Martin *et al*., 1997). On the other hand, hexokinase dependent sugar sensing and signaling can only be triggered by hexoses that are substrates for hexokinase or indirectly by sugars that can be converted into such hexoses (Jang & Sheen, 1994). In addition, hexokinase dependent signaling can be altered by transgenic manipulation of hexokinase activity *in planta* (Jang *et al*., 1997; Xiao, Sheen & Jang, 2000).

Hexokinase dependent sugar signaling

Hexokinases are evolutionary conserved enzymes that catalyze the ATP-dependent phosphorylation of free hexoses such as glucose and fructose into glucose-6-P and fructose-6-P, respectively. Free hexoses in plants are usually produced by degradation of allocated sucrose and stored starch (Fernie, Willmitzer & Trethewey, 2002). The products of hexokinase form a pool of hexose phosphates which are readily inter-converted by reversible enzymatic reactions (Buchanan, Gruissem & Jones, 2000). This hexose phosphate pool is the main

starting material for a variety of biosynthetic pathways as well as for energy production through glycolysis. Thus, hexokinase is needed to make allocated and stored energy available to the metabolic machinery of individual plant cells.

Most plants are believed to have multiple isoforms of hexokinase. For example, the genome of *Arabidopsis thaliana* harbours six apparent hexokinase encoding genes (Rolland, Moore & Sheen, 2002). In addition, *Arabidopsis* and other plant species have distinct enzymes specialized in fructose phosphorylation (Pego & Smeekens, 2000). Some hexokinase encoding genes have been characterized but a comprehensive functional description of all hexose phosphorylating enzymes in one plant is still missing. Different hexose phosphorylating isozymes in a given plant species may potentially have quite different functions. They could differ in their expression, regulation, substrate specificity, tissue distribution and importantly, in their subcellular localization. Hexokinase has long been considered a soluble cytoplasmic enzyme due to its importance for glycolysis. This view has been challenged by biochemical studies describing hexose phosphorylating activities associated with specific cellular compartments including Golgi (Seixas da-Silva, Rezende & Galina, 2001), mitochondria (Cosio & Bustamante, 1984; Wiese *et al*., 1999; Seixas da-Silva, Rezende & Galina, 2001) and chloroplasts (Wiese *et al*., 1999; Miernyk, & Dennis, 1983; Singh *et al*., 1993; Stitt, Bulpin & ap Rees, 1978). The subcellular localization of one plant hexokinase, from spinach, was studied in detail. It was found that this hexokinase is inserted into the outer envelope membrane of chloroplasts, facing the cytoplasmic side (Wiese *et al*., 1999). The membrane integration is mediated by an N-terminal membrane anchor. Based on sequence comparisons, such membrane anchors exist also in many other plant hexokinases, suggesting that insertions into the membranes of different organelles may be relatively common.

As already stated, in addition to their metabolic role, plant hexokinases have been proposed to be involved in hexose sensing and signaling. Such hexokinase dependent sugar signaling can be further subdivided based on whether hexokinase provides a strictly regulatory or a metabolic/catalytic function (Xiao, Sheen & Jang, 2000). In the former case, the signal is independent of the catalytic activity of the enzyme. *Arabidopsis* genes regulated by glucose in this way are exemplified by those encoding chlorophyll a/b-binding protein, plastocyanin and the ribulose-1,5-bisphosphate carboxylase small subunit (Xiao, Sheen & Jang, 2000). The existence of such signaling is supported by the finding that heterologous expression of yeast hexokinase 2 elevates the glucose phosphorylation but still fails to enhance glucose-dependent signaling in *Arabidopsis* (Jang *et al*., 1997). A signaling function for hexokinase and/or one of its products is also suggested by the fact that some of these responses can be triggered by glucose analogues (2-dGlc) which are substrates for hexokinase but which cannot be further metabolized (Jang & Sheen, 1994; Tiessen *et al*., 2003). In contrast, sensing and signaling that is dependent on further metabolism of glucose cannot be triggered by the same glucose analogue (Lejay *et al*., 2003). In such cases, signaling is affected by hexose levels and depends on hexokinase function, but it is likely that a downstream product is the real effector molecule. As a consequence, such signaling can be enhanced also by heterologous expression of yeast hexokinase (Xiao, Sheen & Jang, 2000). *Arabidopsis* genes regulated by sugars in this manner

include *AtNrt2.1* which encodes a NO_3^- transporter and *PR1* and *PR5* encoding pathogenesis-related transcripts (Xiao, Sheen & Jang, 2000; Lejay *et al*., 2003).

It should be emphasized that it is not a simple task to experimentally discriminate between the two proposed roles of hexokinase within hexose sensing. The strictly regulatory sensor function of plant hexokinase has been questioned since much of the evidence described above is circumstantial (Halford *et al*., 1999; Moore & Sheen, 1999; Hardie, 1999). However, it was recently shown that the glucose phosphorylating activity and regulatory function of *Arabidopsis* hexokinase 1 can be separated, something which supports the existence of a distinct glucose sensing function for hexokinase (Moore *et al*., 2003). The general applicability of this finding to other plant hexokinases remains to be proven. It is likely that some hexokinase isozymes may have sensing functions while others provide strictly metabolic functions.

Snf1-related kinases and their roles in metabolic regulation

The eukaryotic family of Snf1-related kinases

As already mentioned, the *SNF1* gene is required for derepression of a large number of genes in response to glucose limitation in the yeast *Saccharomyces cerevisiae*. The gene encodes a serine/threonine protein kinase with an N-terminal catalytic domain followed by a C-terminal regulatory domain (Hardie, Carling & Carlson, 1998). The Snf1 kinase belongs to an evolutionary conserved family of enzymes which appear to exist in all eukaryotes (Figure 4) (Halford & Hardie, 1998). The mammalian counterpart of the Snf1 kinase is called the AMP-activated kinase (AMPK) since it is activated by high AMP/ATP ratios. Once activated, AMPK regulates a number of metabolic enzymes by phosphorylation, the end result of which is less waste of energy and an increased utilization of storage products. Thus, both Snf1 and AMPK are activated in response to limited energy availability and they both cause metabolic changes that alleviate the cause behind their activation, *i.e*. reduced energy levels. The whole family of eukaryotic Snf1/AMPK related kinases has therefore been proposed to share a common function as metabolic sensors or energy gauges (Hardie, Carling & Carlson, 1998).

The first *SNF1*-related kinase (SnRK) sequence from a plant was published in 1991 (Alderson *et al*., 1991) and since then a large number of SnRK sequences from different plants have been described (Halford & Hardie, 1998). Based on sequence comparisons, it is clear that they fall into three conserved subfamilies referred to as SnRK1, SnRK2 and SnRK3 (Halford & Hardie, 1998). The SnRK1 subfamily appears to be the true homologues of yeast *SNF1* and mammalian AMPK while SnRK2 and SnRK3 are more divergent plant specific subgroups. The SnRK1 subgroup can be further divided into SnRK1a which are represented in all plants and SnRK1b which appear to be specific to cereals (Halford *et al*., 2003). Plants appear to have several potentially redundant copies of genes within each subgroup. For example, the *Arabidopsis* genome harbours three SnRK1 genes, 10 SnRK2 genes and 29 SnRK3 genes (Halford *et al*., 2003).

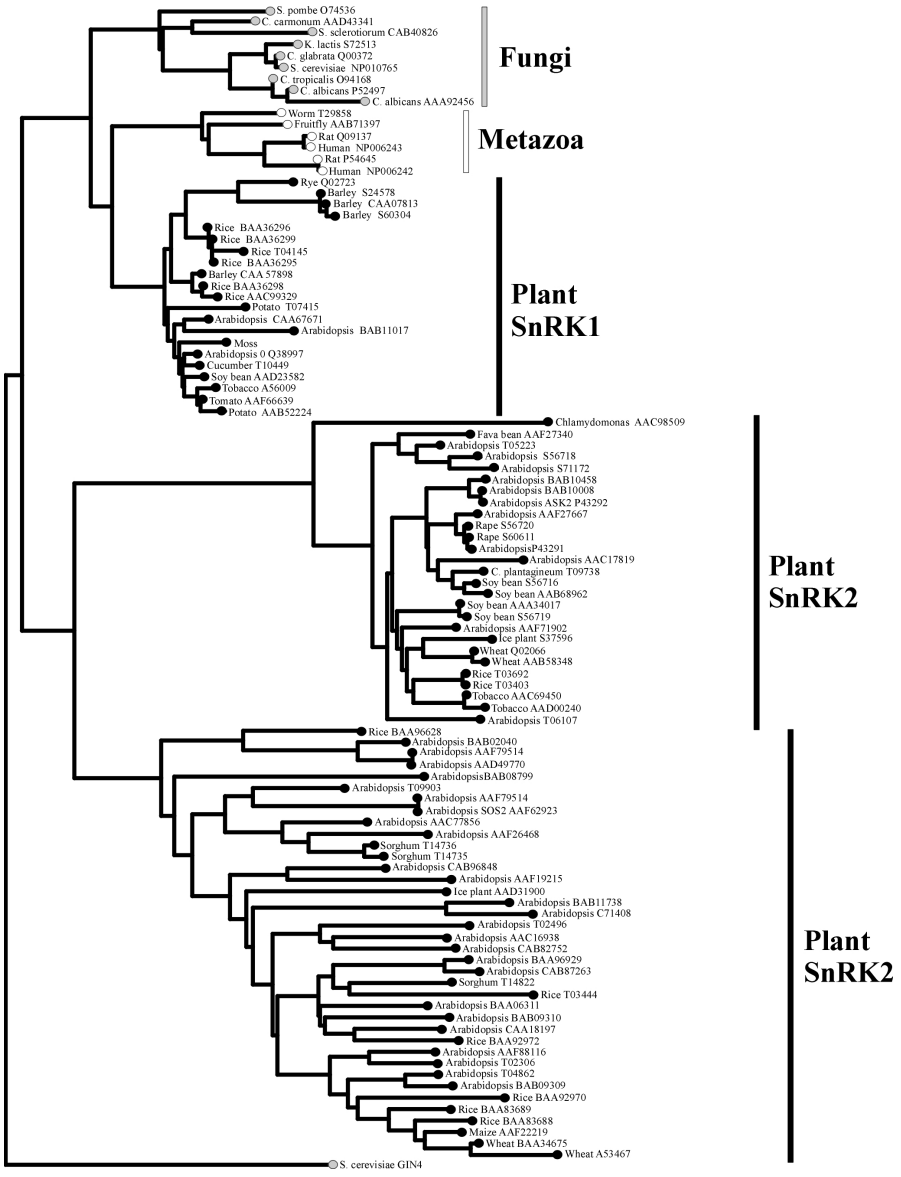

Figure 4. Phylogenetic tree showing Snf1-related kinases. The tree is based on an amino acid sequence alignment done in the ClustalX software with default settings (Thompson *et al*., 1997). The yeast Gin4 kinase was included as an outgroup.

Biochemical characterization of Snf- related kinases

All Snf1-related kinases have highly conserved catalytic domains and share similar target specificities. Rat acetyl-CoA carboxylase is a known substrate of AMPK and the target sequence around the phosphorylation site was used to design a synthetic target peptide (Davies *et al*., 1989). The resulting SAMS peptide has been useful as a synthetic substrate in the characterization of Snf1-related kinases not only from animals but also from yeast and plants. SAMS phosphorylating activity in plants was first described by Mackintosh *et al* (1992) and since then, the SAMS peptide and the related AMARA peptide (Dale *et al*., 1995) have been used in combination with SnRK1 specific antibodies to purify and characterize a number of plant Snf1-related enzymes (Ball *et al*., 1994; Ball *et al*., 1995; Barker *et al*., 1996; Man *et al*., 1997; Sugden *et al*., 1999a; Sugden *et al*., 1999b; Crawford *et al*., 2001). One important contribution to the elucidation of SnRK1 function in plants was the identification of several enzymes that are targets for SnRK1-dependent phosphorylation, including HMG-CoA reductase, sucrose phosphate synthase and nitrate reductase (Sugden *et al*., 1999a). This suggests that SnRK1, in analogy with mammalian AMPK, may regulate several biosynthetic pathways including isoprenoid synthesis, sucrose synthesis and nitrogen assimilation by direct phosphorylation of key enzymes.

Subunit composition of Snf1-related kinases

The yeast Snf1 kinase is a heterotrimeric complex made up of a catalytic α-subunit encoded by the *SNF1* gene, a β-subunit encoded by the partially redundant *SIP1*, *SIP2* and *GAL83* genes, and a γ-subunit encoded by the *SNF4* gene (Jiang and Carlson, 1997). A model has been proposed for how Snf1 is regulated by the carbon source in which the C-terminal regulatory domain binds to the kinase domain and blocks its active site in the presence of glucose. During glucose depletion, the regulatory Snf4 subunit binds to the Snf1 regulatory domain, thus preventing it from blocking the active site (Jiang & Carlson, 1996).

A heterotrimeric complex similar to that in yeast has been proposed also for plant Snf1-related kinases based on the identification and characterization of SnRK1 interacting proteins. These include both β-subunits (Lakatos *et al*., 1999; Bouly *et al*., 1999; Ferrando *et al*., 2001), γ-subunits (Bouly *et al*., 1999; Kleinow *et al*., 2000; Slocombe *et al*., 2002) and a novel class of proteins carrying β- and γ-motifs in combination (Lumbreras *et al*., 2001). Anti-sense silencing of the sugar repressible potato β-subunit *StubGAL83* was shown by Lovas *et al*. (2003) to result in disturbed root and tuber development. Otherwise, functional characterization *in planta* of isolated β- and γ-subunits has so far largely been limited to expression and copy number studies. The existence of multiple isoforms of each subunit in a plant makes it possible that differential complex composition contributes to functional variation. This is indeed the case in yeast, where different β-subunits affect both subcellular localization (Vincent *et al*., 2001) and function of the enzyme (Vyas *et al*., 2003).

Regulation of Snf- related kinase activity by energy availability

Mammalian AMPK is allosterically activated by high AMP/ATP ratios (Hardie, Carling & Carlson, 1998 and references therein). AMPK is also regulated by reversible phosphorylation which in turn is also affected by AMP. Thus, AMPKK, the biochemically defined upstream kinase kinase regulating AMPK is activated by AMP and furthermore, the binding of AMP to AMPK makes it a better substrate for phosphorylation but a worse substrate for dephosphorylation. Yeast Snf1 and plant SnRK1 also appear to be regulated by reversible phosphorylation since they can be reactivated by the addition of partially purified mammalian AMPKK following phosphatase inactivation (Woods *et al*., 1994; Sugden *et al*., 1999). The gene(s) encoding the mammalian AMPKK remain(s) elusive but three redundant yeast genes encoding Snf1-phosphorylating kinases were recently described (Hong *et al*., 2003). The genes encoding plant SnRK1 phosphorylating and dephosphorylating enzymes remain to be described. In contrast to mammalian AMPK, yeast Snf1 and plant SnRK1 do not appear to be allosterically regulated by AMP (Wilson, Hawley & Hardie, 1996; Mackintosh *et al*., 1992). This does not exclude other mechanisms for AMP regulation of SnRK1 activity and Sugden and colleagues (1999) have indeed shown that SnRK1 inactivation by PP2C mediated dephosphorylation is inhibited by physiological amounts of AMP. It should be emphasized that high intracellular AMP is an indicator of a low cellular energy status. The negative effect of AMP on SnRK1 dephosphorylation therefore suggest that also plant SnRK1 kinases, in analogy with yeast Snf1 and mammalian AMPK, are activated in response to a reduced energy supply. Another mechanism that may contribute to such regulation is the proposed allosteric inhibition of SnRK1 by glucose-6-P (Toroser, Plaut & Huber, 2000).

Plant Snf1-related kinase interacting proteins

A number of yeast two hybrid (YTH) screens for SnRK1 interacting proteins have been performed. In addition to the β and γ subunits discussed above, a few other interactors of potential functional importance have also been described. These include an *Arabidopsis* WD40 protein called Prl1 which has been shown to interact with *Arabidopsis* SnRK1 proteins AKIN10 and AKIN11 both in a YTH assay and *in vitro* (Bhalerao *et al*., 1999). Interestingly, a *prl1* mutant suffers from a number of pleiotropic phenotypes including derepression of sucrose regulated gene expression (Nemeth *et al*., 1998). SnRK1 activity was elevated in the *prl1* mutant suggesting that Prl1 could be a negative regulator of the SnRK1 enzymes (Bhalerao *et al*., 1999).

Arabidopsis SnRK1 proteins AKIN10 and AKIN11 have also been shown to interact with SKP1/ASK1 which is a homologue of the yeast SCF component SKP1, implicated in the yeast glucose induction pathway (Farras *et al*., 2001). Furthermore, SKP1/ASK1 itself was also used as the bait in a YTH screen that resulted in the isolation of a number of genes encoding F-box proteins related to yeast Grr1 (Farras *et al*., 2001). Further support for a functional link between SnRK1 and proteasome mediated proteolysis comes from the fact that the proteasome subunit α/PAD4 was shown to interact with AKIN10 and AKIN11 (Farras *et al*., 2001).

Other SnRK1 interactors of potential functional importance include a protein tyrosine phosphatase (Fordham-Skelton *et al*., 2002) and viral proteins known to enhance susceptibility of host plants (Hao *et al*., 2003). Interestingly, Halford *et al*. (2003) have also reported that an AP2 transcription factor interacts with SnRK1. This could reflect a very direct mechanism for SnRK1 involvement in transcriptional regulation but the details and significance of this interaction remain to be described.

Genetic studies of Snf1-related kinase function in planta

In addition to the biochemical characterization discussed above, functional SnRK1 studies have relied on a number of reverse genetic approaches. Thus, silencing of a potato SnRK1 kinase was shown to result in decreased sucrose synthase expression and a lack of sucrose induction of the sucrose synthase gene (Purcell *et al*., 1998). Furthermore, SnRK1 antisense silencing in barley resulted in non functional pollen which could be due to an inability to store starch (Zhang *et al*., 2001). Results from SnRK1 antisense experiments in wheat embryos also suggest a function for SnRK1 in relieving sugar repression of α-amylase when sugar levels are low (Lauire *et al*., 2003). These studies suggest that plant SnRK1 may be important for the induction/derepression of genes needed during conditions of energy starvation. Thus, SnRK1 is needed for sucrose synthase expression (Purcell *et al*., 1998) and possibly also invertase (Halford *et al*., 2003), which would accelerate the degradation of sucrose. SnRK1 is also needed for α-amylase expression (Laurie *et al*., 2003) that will facilitate the breakdown of stored starch. Increasing the capacity of sucrose degradation is only meaningful if there is sucrose available and accordingly, sucrose synthase expression is also positively regulated by sucrose (Fu and Park, 1995; Purcell *et al*., 1998). Halford and Dickinson (2001) have proposed that SnRK1 may be important also for this sucrose induction and thus may respond to a combination of low glucose and high sucrose. The latter is supported by recent findings by Tiessen *et al*. (2003) where the sucrose specific redox activation of potato tuber AGPase activity was shown to depend on SnRK1 function.

The reverse genetic studies discussed above were all limited either to tissue specific expression of transgenes (Purcell *et al*., 1998; Zhang *et al*., 2001) or transient experiments involving a specific tissue type (Laurie *et al*., 2003). There is only one report on the successful generation of whole plants with compromised SnRK1 activity (Hao *et al*., 2003). In addition, there is also one report on the problems of generating such potato plants, possibly because SnRK1 may encode an essential function (Halford *et al*., 1994). Hao and colleagues (2003) expressed a SnRK1 antisense construct from a constitutive promoter and managed to regenerate plants. They showed an enhanced susceptibility of SnRK1 silenced plants to viral infections, but otherwise no morphological phenotypes were reported. The experiments were performed in tobacco but since the antisense construct was based on *Arabidopsis AKIN11* and no decrease in the native sense SnRK1 transcripts or SAMS phosphorylating activity was demonstrated, it is not clear to what degree SnRK1 was silenced. Therefore, although the effects on virus susceptibility are very convincing, it is hard to draw any conclusions about other *in vivo* functions of SnRK1 from this experiment.

Physcomitrella patens as a plant model system

Homologous recombination and gene targeting

There are many ways, both direct and indirect, to study the functions of isolated genes (Osterlund & Paterson, 2002). Among others, these include sequence comparisons, expression profiling and over-expression. The two former approaches only provide circumstantial evidence while the latter, since it is based on non-physiological expression levels, often produce effects that are difficult to interpret. A more reliable way to study gene function, which is both direct and physiological, is to use reverse genetics. This is a collective term for methods that reduce or abolish the expression of a specific gene. Expression of the gene can be inhibited by anti-sense silencing, or the gene itself can be destroyed. The latter can be achieved by classical mutagenesis, transposon mutagenesis, or gene targeting (Schaefer, 2002). Gene targeting differs from the other two methods by being specific for the gene of interest. It was first described in yeast (Orr-Weaver, Szostak & Rothstein, 1981) and is based on sequence specific recombination between foreign and genomic DNA. Gene targeting can therefore only be used in systems with an efficient native system for homologous recombination. This includes the yeast *Saccharomyces cerevisisae* and mouse embryonic stem cells. In addition, the moss *Physcomitrella patens* holds a unique position as the only land plant in which homologous recombination is efficient enough to support gene targeting (Schaefer , 2002).

The moss Physcomitrella patens

Physcomitrella patens and other mosses belong to a group of simple plants called bryophytes which also includes liverworts and hornworts. These three groups of plants are quite different but still have some important features in common which distinguish them from higher plants (Raven, Evert & Eichhorn, 1992). Thus, although some genera have specialized conducting cells, bryophytes generally lack a true vascular system. Furthermore, they are attached to the substrate through a filamentous tissue type called rhizoids which are believed to mainly serve structural purposes. Finally, unlike most other plants, the main vegetative growth form of bryophytes is haploid. Thus, the haploid gametophyte is the dominant growth phase while the diploid sporophyte is always attached to, and often highly dependent on, the gametophyte. This haploid dominance of bryophytes further enhances the suitability of *Physcomitrella* for reverse genetic studies since it makes it possible to examine the phenotypes that result from recessive loss of function mutations such as targeted gene knockouts, without first obtaining homozygous diploids by backcrosses.

Physcomitrella is a short-lived opportunist which is normally found in open disturbed habitats (Schaefer & Zrÿd, 2001). The wild type strain used by most laboratories was isolated in England but *Physcomitrella* is widespread and has also been described at locations near Uppsala (Berlin & Isaksson, 2002). It is easily cultivated *in vitro* at a low cost and many molecular genetic tools such as vectors, promoters and selection markers from higher plants can be used without further modifications (Cove, Knight & Lamparter, 1997; Reski, 1999). *Physcomitrella* is also relatively easy to transform by PEG mediated DNA uptake into protoplasts,

which produces independent clones immediately following regeneration (Schaefer *et al*., 1991).

Mosses are believed to have diverged from the vascular plants more than 500 million years ago and currently comprise some 10,000 species (Schaefer & Zrÿd, 2001). Plant research of today is largely focused on flowering plants such as the model plant *Arabidopsis thaliana*. Much research is also being done on economically important crops such as rice, maize, potato, tomato and different cereals. These are all, in evolutionary terms, closely related angiosperms. A model system such as *Physcomitrella* can therefore also provide important information as a missing evolutionary link between green algae and higher plants.

The Physcomitrella life cycle

Physcomitrella has a relatively simple physiology but it still has specific cell and tissue types that resemble those of higher plants (Figure 5). The life cycle and the different cell and tissue types of *Physcomitrella patens* have been described in detail by Reski (1998). The gametophyte part of the life cycle can be divided into juvenile two-dimensional protonemal growth and adult upright gametophore growth. The tissue type that first emerges from regenerating spores is the filamentous protonemata. Protonemal filaments grow by apical cell divisions and consist of two distinct but inter-convertible cell types, chloronemata and caulonemata. Chloronemal cells are relatively short with perpendicular cross walls and numerous well developed chloroplasts. Caulonemal cells differ from chloronemal cells by being generally thinner, longer, faster growing and having oblique cross walls and fewer and smaller chloroplasts. Protonemal filaments are subjects to sub-apical branching and caulonemal side branch initials occasionally develop into buds. The protonemal bud serves as the meristem initial needed to generate the upright leafy shoot or gametophore. Three faced apical cell divisions give rise to the non vascular stem which is surrounded by leaves that are one cell layer thick. A number of root-like filamentous rhizoids emerge from the base of the gametophore. The leafy gametophores bear the sex organs, the male antheridia and the female archegonia. The mature antheridia release spermatozoids which need a surface film of water to swim down the neck of the archegonia in order to reach and fertilize the egg. Unlike some other moss species, *Physcomitrella* is monoecious which means that each individual have both male and female sex organs and can self fertilize. After fertilization, the zygote grows by cell divisions to develop the diploid sporophyte consisting of a short stalk (seta) carrying a spore capsule. Meiosis occurs within the spore capsule, and a mature spore capsule contains some 5000 haploid spores which, given the right circumstances, can regenerate and form a new haploid plant.

Importantly, the growth and development summarized above appears to be regulated by the same factors that regulate higher plant growth and development. These include light, temperature, stress, nutrient availability, gravity and phytohormones such as auxins, cytokinins and abscisic acid (Reski 1998 and references therein). Several reports, some based on the manipulation of signaling pathways by gene targeting, have furthermore suggested that the molecular mechanisms for sensing and signaling to a large extent are conserved between

mosses and higher plants (Knight *et al*., 1995; Imaizumi *et al*., 2002). *Physcomitrella patens* may therefore be a handy model system for the investigation of how plant cells sense and respond to certain changes in their close environement.

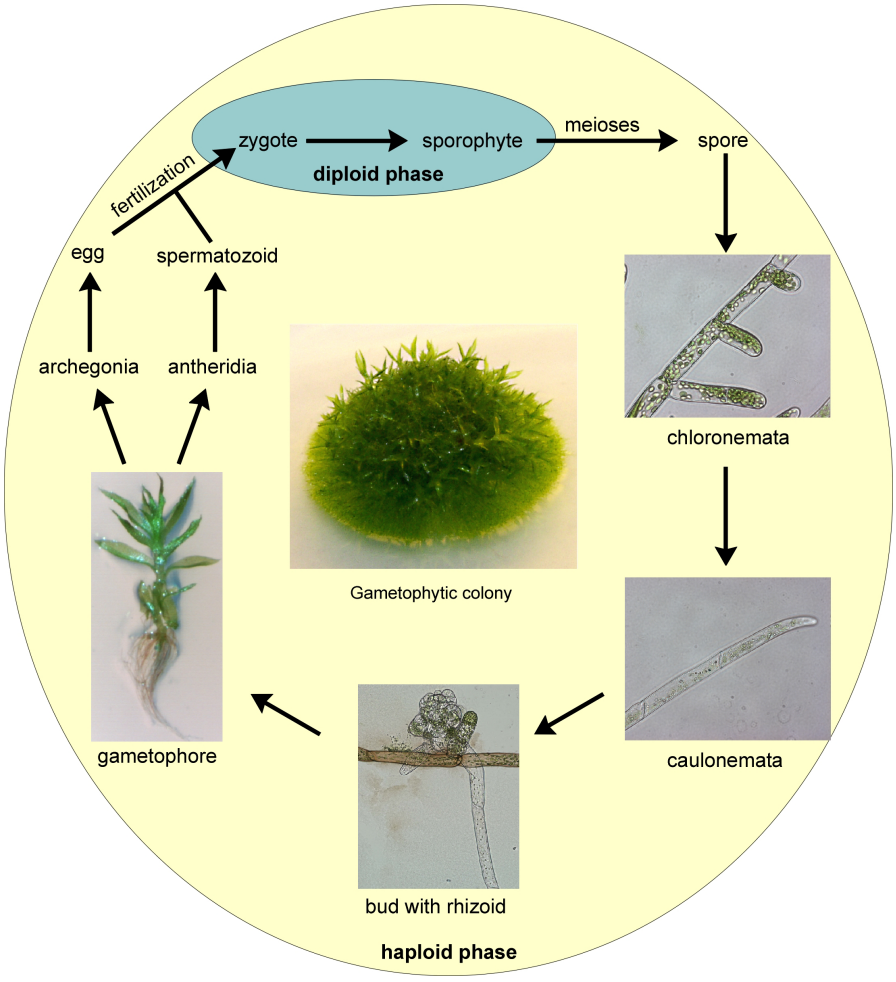

Figure 5. The life cycle of *Physcomitrella patens*.

Physcomitrella genomics

The haploid genome of *Physcomitrella patens* consists of 480 Mbp of DNA distributed on 27 small chromosomes (Reski, 1998; Reski *et al*., 1994). A coordinated *Physcomitrella* genome sequencing project is still pending and detailed information of the organization of the genome is therefore limited. Based on the cases where both genomic and cDNA sequences from one gene are available, it appears that the organization of exons and introns is similar to that in higher plants (Schaefer, 2002). However, introns appear to be generally larger in *Physcomitrella* than in *Arabidopsis*, which in part may explain the difference in genome size. A number of *Physcomitrella* EST sequencing projects has been undertaken and resulted in considerable knowledge about the coding regions of the genome (Reski *et al*., 1998; Machuka *et al*., 1999; www.moss.leeds.ac.uk; Rensing *et al*., 2002; Nishiyama *et al*. 2003). Nishiyama and colleagues (2003) have recently summarized conclusions extracted from the analyses of 102,885 publicly available EST sequences. They conclude that there are at least 15,883 putative transcripts expressed in *Physcomitrella* and that a minimum of 66.4% of the 26,174 amino acid sequences encoded by the *Arabidopsis* genome have clear homologues in *Physcomitrella*. These figures are clearly understood to be underestimates since the *Physcomitrella* EST collection was found not to be saturated.

Aims of the present investigation

The general aim of my work has been to contribute to the understanding on how energy availability affects the metabolism, growth and development of plants. In particular, my work has been focused on regulatory effects caused by carbohydrates such as sugars. Significantly, some of the underlying mechanisms mediating such effects appear to be partly conserved between yeast and plants. These mechanisms are comparably well understood in yeast and we therefore used this model organism as a platform to learn more about sugar metabolism and signaling in plants.

In the first part of my work, a genetic screen was performed in yeast aimed at the isolation of *Arabidopsis thaliana* cDNAs encoding proteins of potential interest for sugar sensing and signaling (I).

In the second part of my work, plant counterparts to key components in the yeast glucose repression pathway were cloned and characterized in the moss *Physcomitrella patens*. This included one hexokinase (II & IV) and two functionally redundant Snf1-related kinases (III). In order to elucidate the *in vivo* function of these proteins, the corresponding genes were knocked out by gene targeting and the resulting mutants were analyzed in detail.

Results and discussion

Cloning of *AtGRH1* by pathway activation in yeast (I)

The yeast *GAL1* gene encodes a galactokinase which is needed for growth on galactose but which is dispensable for growth on glucose (Lohr, Venkov & Zlatanova, 1995). It therefore makes sense that *GAL1* expression is galactose induced and glucose repressed. Glucose repression of the *GAL1* gene is mediated by the Mig1 repressor (Nehlin, Carlberg & Ronne, 1991). The name Mig1 comes from the isolation of the corresponding gene as a Multicopy Inhibitor of *GAL1* (Nehlin & Ronne, 1990). Thus, even though Mig1 is normally active only in the presence of glucose, over-expression causes it to repress target genes such as *GAL1* also in the absence of glucose.

The Mig1 repressor is an effector molecule in the complex network of glucose sensing and signaling mechanisms that bring about changes in gene expression (Johnston, 1999; Rolland, Winderickx & Thevelein, 2002). The genetic screen presented in paper I was based on the assumption that overexpression also of other components in this network could result in constitutive glucose repression of the *GAL1* promoter. If so, it should be possible to isolate functional homologues of these components from other species by using their ability to inhibit the *GAL1* promoter when overexpressed in yeast, provided that the signaling pathway (in this case glucose repression) is, at least in part, conserved and thus can be activated by heterologous proteins.

We decided to test this approach by screening an *Arabidopsis* cDNA library in a yeast expression vector (Minet, Dufour & Lacroute, 1992) for clones that could inhibit *GAL1* expression by activating glucose repression when expressed in yeast. Thus, the yeast strain D69 was transformed with a multicopy library expressing *Arabidopsis* cDNAs from a strong constitutive yeast promoter. The yeast strain D69 has an integrated copy of a chimeric construct in which the *GAL1* promoter is fused to the *TPK2* gene encoding the cAMP dependent protein kinase (Nehlin, Carlberg & Ronne, 1989). High level expression of the *TPK2* gene is lethal and since the strong *GAL1* promoter is active on galactose, this strain is normally unable to grow on this carbon source. By shifting transformed cells from glucose to galactose, we could identify transformants expressing *Arabidopsis* cDNAs which could prevent the lethal expression of *TPK2*.

Some 50,000 yeast transformants were screened which resulted in the isolation of 6 independent suppressor plasmids. These were rescued, retested and then sequenced. As outlined above, our primary aim with this screen was to isolate cDNAs homologous to components in the yeast glucose sensing and signaling machinery. In order to determine which of the six clones, if any, that were isolated due to their ability to activate glucose repression, we next tested whether their suppression was dependent on Mig1. One of the six clones was clearly dependent on Mig1 as proven by a loss of suppression in a D69 derivative deleted for *MIG1*. In contrast, the effect mediated by the other five clones was independent of Mig1 which suggests alternative suppression mechanisms. Obvious candidate

mechanisms are interference with galactose induction of the *GAL1* promoter or interference with the function of the *TPK2* gene product. However, the isolation of at least one clone which could activate glucose repression in yeast confirmed the general validity of the cloning by pathway activation approach.

Sequencing of the one clone proven to activate Mig1 mediated repression of the *GAL1* promoter showed that the insert was highly homologous to the yeast GRR1 gene, and we therefore named it *AtGRH1* (*GRR1* Homologous 1). Yeast *GRR1* got its name because the corresponding mutant is Glucose Repression Resistant (Flick & Johnston, 1991). Given this mutant phenotype, it is not unreasonable to expect that overexpression of yeast *GRR1* could result in the opposite effect, namely constitutive glucose repression. We were indeed able to verify this hypothesis by showing that also yeast *GRR1* could suppress the growth inability on galactose of the yeast strain D69 in a *MIG1* dependent manner.

As described in the introduction, yeast Grr1 exerts its main function in the glucose induction pathway in which it is needed for glucose induction of hexose transporter expression. Its requirement for the glucose repression pathway is therefore believed to be indirect. According to this interpretation, the lack of glucose transporter expression in a *grr1* mutant results in decreased intracellular glucose levels leading to less stringent glucose repression. It cannot be excluded, though, that *GRR1* also plays a more direct role in the glucose repression pathway.

Yeast Grr1 belongs to the F-box proteins which all share a common function within ubiquitin-dependent proteolysis (Patton, Willems & Tyers, 1998). As already discussed, F-box proteins are part of large protein SCF complexes which label protein substrates for proteasome mediated proteolysis by polyubiquitinylation. F-box proteins are believed to act as the specificity determinants of these complexes and in addition to their F-box, they also possess various protein protein interaction domains for substrate recruitment. For example, yeast Grr1 possess numerous leucine rich repeats and has been suggested to affect glucose induction by mediating glucose induced degradation of Mth1 (Flick *et al*., 2003). Mth1 in turns is needed for the maintenance of Rgt1 repression of hexose transporter genes.

Yeast Grr1 is a considerably larger protein than AtGrh1 (1151 vs. 585 residues) but they share a common organization where an N-terminal F-box is followed by a large number of leucine rich repeats. The F-box motif is known to mediate physical interaction with the rest of the SCF complex through an evolutionary conserved protein called Skp1 (Patton, Willems & Tyers, 1998). Interestingly, two *Arabidopsis* Skp1 homologues have been shown to be able to relieve Mig1 repression when expressed in yeast (Schouten *et al*., 2000). Heterologous expression of *AtSKP1a/b* and *AtGRH1* therefore affect the same yeast signaling pathway but in opposite ways. This could indicate a functional relationship also *in planta*. By using the yeast two hybrid assay we could show that AtGrh1 is capable of interacting with both AtSkp1a and AtSkp1b which strengthens this hypothesis.

The fact that *AtSKP1a/b* and *AtGRH1* both affect sugar regulated gene expression in yeast could imply that they provide a similar function in *Arabidopsis*, though this remains to be proven. In this context it should be

mentioned though, that AtTir1, the closest relative of AtGrh1, has been implicated in the auxin signaling pathway (Ruegger *et al*., 1998). Furthermore, the *Arabidopsis* genome has been claimed to contain 10 cullin genes, 21 SKP1 genes and no less than 703 genes encoding F-box proteins (Risseeuw *et al*., 2003). This wealth of SCF components offers an enormous combinatorial capacity suggesting that ubiquitin mediated proteolysis might be involved in numerous processes in planta. This notion is supported by the diversity of functions assigned to *Arabidopsis* F-box proteins. Mutant analysis has implicated F-box proteins in auxin signaling, flower development, jasmonic acid signaling, circadian clock, light signaling and senescence (Dieterle *et al*., 2001; Ingram *et al*., 1997; Nelson *et al*., 2000; Ruegger *et al*., 1998; Somers *et al*., 2000; Stirnberg, van De Sande & Leyser, 2002; Woo *et al*., 2001; Xie *et al*., 1998).

Cloning and characterization of a novel chloroplast stromal hexokinase from the moss *Physcomitrella patens* (II & IV)

In order to learn more about the metabolic and regulatory functions of hexokinase in plants, we initiated a study of hexokinase in the moss *Physcomitrella patens* where gene function can be studied by targeted knockouts (Schaefer , 2002).

We used a combination of degenerative PCR, RACE PCR and conventional PCR to obtain full length cDNA and genomic clones of a hexokinase gene which was named *PpHXK1*. In addition, we also cloned a second gene called *PpHXK2* which has not yet been studied in any detail. A low stringency Southern blot indicated that *PpHXK1* is a single copy gene although a faint additional band visible in some lanes indicated the existence of at least one more distantly related hexokinase gene. This gene could be *PpHXK2* or another yet unidentified gene. The transcription of *PpHXK1* was shown by quantitative RT-PCR to be essentially constant in protonemal tissue, and not affected by changes in light intensity or the addition of external glucose.

Interestingly, PpHxk1 was predicted by the targetP software (Emanuelsson *et al*, 2000) to contain an N-terminal 37 amino acid residue chloroplast targeting sequence. Such peptide signals mediate targeting of nuclear encoded proteins for uptake into organelles such mitochondria and chloroplasts (Emanuelsson & von Heijne, 2001). We were able to verify the predicted chloroplast localization of PpHxk1 by making translational fusions to the green fluorescent protein (GFP). Thus, while GFP alone was found in the cytoplasm, GFP fused to PpHxk1 was exclusively found in the chloroplasts. Moreover, this localization was strictly dependent on the 37 amino acid residue targeting peptide.

Two types of chloroplast associated hexokinase activities have previously been described. First, glucose and fructose phosphorylating activities have been found in the chloroplast stroma (Miernyk, & Dennis, 1983; Singh *et al*., 1993). These have only been biochemically defined, and a gene encoding such an enzyme has not yet been described in any plant. Second, the spinach hexokinase SoHxK1 was shown to be associated with the outer envelope of chloroplasts, facing the cytoplasmic side (Wiese *et al*., 1999). This localization was further shown to be mediated by an N-terminal hydrophobic membrane anchor.

We next knocked out the *PpHXK1* gene by gene targeting. Significantly, the glucose and fructose phosphorylating activities of the resulting *hxk1* mutant were strongly reduced, to 22% and 53% of the wild type levels, respectively. This reduction in hexose phosphorylating activity provided us with a way to confirm and further pinpoint the subcellular localization of PpHxk1. Thus, we purified intact *Physcomitrella* chloroplasts from both the wild type and the *hxk1* mutant and examined the localization of glucose phosphorylating activity within the organelles. Wild type chloroplast associated hexokinase activity was found divided between a major soluble stromal fraction and a small membrane associated fraction. Interestingly, the latter fraction was not affected in the *hxk1* mutant while the soluble stromal activity was abolished. This proves that PpHxk1 is localized in the chloroplast stroma while an envelope association as suggested for spinach SoHxK1 could be excluded (Wiese *et al*., 1999). *PpHXK1* is therefore the first example of a gene shown to encode a chloroplast stromal hexokinase activity. The fact that PpHxk1 is the major hexokinase in *Physcomitrella* further suggests that hexose phosphorylation inside the chloroplasts may be more physiologically important than previously realized.

A detailed characterization of the *hxk1* mutant showed that it suffered from a number of growth phenotypes including a decrease in the formation of caulonemal filaments and increased chloronemal branching. A similar growth pattern is observed also in the wild type following energy starvation as accomplished by a reduction in light supply. This suggests that the mutant suffers from energy starvation. The mutant is also hypersensitive to the phytohormones cytokinin and ABA, something which emphasizes similarities to *Arabidopsis*. Endogenous ABA levels were not affected in the mutant and its increased ABA sensitivity is therefore unlikely to be due to enhanced ABA synthesis or reduced ABA degradation. Instead, it is more likely explained by changes in the detection or transduction of the ABA signal.

Arabidopsis hexokinase 1 has been suggested to provide an important function as a glucose sensor (Moore *et al*., 2003). Thus, an *Arabidopsis* hexokinase 1 mutant has complex growth phenotypes and is deficient in glucose regulation of gene expression. These phenotypes could be complemented by expression of a catalytically inactive mutant kinase. This suggests that the role of *Arabidopsis* hexokinase 1 within glucose sensing is independent of the catalytic/metabolic function of the enzyme. We believe that a signaling function for PpHxk1 is unlikely for two reasons. First, the chloroplast stromal localization of PpHxk1 makes it less likely to be involved in cytoplasmic-nuclear signal transduction. Second, even though the *hxk1* mutant appears to suffer from an energy starvation phenotype, it is still capable of responding to both external glucose and light with the same relative efficiency as the wild type.

A comparison of all available plant hexokinase sequences revealed that they can be grouped into two types, depending on their N-termini. One group, named Type A by us, has predicted N-terminal chloroplast target peptides similar to PpHxk1. It is therefore likely that Type A hexokinases from higher plants also are chloroplast stromal enzymes, though this remains to be proven. A second group of plant hexokinases, named Type B by us, comprise the classical membrane-associated

hexokinases that have been studied in higher plants (Wiese *et al*., 1999; Jang *et al*., 1997; Moore *et al*., 2003; Veramendi *et al*., 1999; Veramendi *et al*., 2002). These hexokinases all share a highly conserved N-terminal hydrophobic membrane anchor. Importantly, *Physcomitrella* also has at least one Type B hexokinase, the product of the *PpHXK2* gene. We therefore conclude that both types of hexokinases, stromal and membrane-associated, appear to be present in all plants.

We further examined how the different types of plant hexokinases are related to each other. For this, we used a phylogenetic tree calculated from the core regions of the enzymes, in order to avoid any bias derived from the N-termini. We found that the Type A hexokinases are present in several deep branches of the tree, which suggests that they represent an ancestral form of plant hexokinase. In contrast, the Type B enzymes are restricted to two well-defined branches within the tree. One branch, termed B1 by us, seems to be very old since it predates the separation of mosses from higher plants. The second branch, B2, comprises all the membrane-associated hexokinases studied in higher plants, such as AtHxk1 and SoHxk1. This branch seems to be of a more recent origin, indicating that it may have arisen by ectopic transfer of the membrane anchor from the older B1 branch.

Cloning and characterization of two genes encoding Snf1-related kinases in the moss *Physcomitrella patens* (III)

We initiated this part of the investigation by cloning both cDNA and genomic sequences from two *Physcomitrella SNF1*-related kinase genes by the same methods used to clone *PpHXK1*. Based on their predicted protein sequences, we could conclude that they were *SNF1*-related kinases of the SnRK1 subfamily and accordingly, they were named *PpSNF1a* and *PpSNF1b*. Expression studies in yeast revealed that both *PpSNF1a* and *PpSNF1b* were able to partly complement a *snf1* mutation in a manner that required the γ-subunit Snf4. Southern blot analysis showed that *PpSNF1a* and *PpSNF1b* are the only two members of a small gene family. We were unable to reproducibly detect *PpSNF1a* and *PpSNF1b* expression by conventional Northern blotting, suggesting that both genes were expressed at very low levels in protonemal tissue. Using quantitative RT-PCR, we could show that the expression of both genes is unaffected by light and external glucose. Transcription of *PpSNF1a* and *PpSNF1b* is therefore unlikely to be regulated by energy abundance.

We next used gene targeting to generate single and double knockout mutants for the two genes. The resulting mutants were tested for SAMS peptide phosphorylation (Davies *et al*., 1989) which is a relatively specific mean of measuring SnRK1 kinase activity. The results clearly showed that PpSnf1a and PpSnf1b both possess SnRK1 kinase activity and furthermore, that they are the only two enzymes with SnRK1 activity in protonemal tissue. Thus, both single mutants had reduced SnRK1 activities, and we were unable to detect any activity at all in the *snf1a snf1b* double mutant. The *snf1a snf1b* double knock-out mutant is to our knowledge the first animal or plant which is completely devoid of SAMS phosphorylating activity.

Phenotypic characterization of the knockout mutants showed that *PpSNF1a* and *PpSNF1b* have redundant functions *in vivo*. Thus, while the double mutant suffers from severe pleiotropic phenotypes, neither single mutant differs from the wild type, except for the partially reduced SnRK1 activity. Such functional redundancy may exist also in higher plants which have multiple SnRK1-encoding genes. For example, *Arabidopsis* have three SnRK1 encoding genes while barley has been estimated to posses between 10 and 20 copies (Halford *et al*., 2003). We further tried to overexpress the *PpSNF1a* gene from the constitutive 35S promoter, but were unable to obtain moss transformants with this plasmid. To our knowledge, there is only one published report on the overexpression of a SnRK1 kinase *in vivo* (Hao *et al*., 2003). In that report it was stated that the overexpressing plants were hard to regenerate and grew more slowly than the wild type. SnRK1 overexpression therefore seems to be harmful to plants. In this context it should be mentioned, however, that overexpression of protein kinases frequently is lethal (Nehlin *et al*., 1989) since run-away phosphorylation will deplete the ATP pool.

The most obvious phenotype of the *snf1a snf1b* double mutant is an excess of caulonemal filaments at the expense of the photosynthetically more active chloronemal filaments. This shift in balance between the two types of protonemal cells is essentially the opposite of the energy starvation phenotype seen in the mutant deleted for *PpHXK1* (II & IV). Thus, in contrast to the *hxk1* mutant, the *snf1a snf1b* double mutant may be suffering from a constitutive high energy mode of growth. This notion is in line with the proposed conserved function of Snf1-related kinases in sensing and responding to conditions of energy starvation (Hardie, Carling & Carlson, 1998). We also observed that the gametophores of the double mutant had an excess of rhizoids while leafy shoots were severely stunted. Rhizoids resemble caulonemal cells by having fewer and poorly developed chloroplasts while chloronemal filaments resemble leafy shoot cells in that they contain numerous green chloroplasts. The impact of energy availability on the balance between rhizoids and leafy shoots has not yet been investigated, but it is not unreasonable to suggest that also this phenotype of the *snf1a snf1b* mutant may reflect a high energy mode of growth and development. In addition to these developmental phenotypes, we also found the double mutant to be hypersensitive to the toxic effects of auxin while it was insensitive to cytokinin. Auxin hypersensitivity correlates well with the developmental phenotypes since auxin is known to induce both caulonemata and rhizoids, while high amounts inhibit the growth of chloronemal filaments and leafy shoots (Ashton, Grimsely & Cove, 1979; Sakaibara *et al*., 2003). One possible interpretation of all these phenotypes is that auxin signaling induces a high energy mode of growth and development which is normally suppressed by low energy in a manner that requires *PpSNF1a* or *PpSNF1b*.

The most exciting phenotype of the *snf1a snf1b* double mutant is its inability to grow in low light or in a normal day/night light cycle. Both these effects could be suppressed by the addition of external glucose, suggesting that they were not simply due to a light signaling defect. Also these phenotypes could be explained by a constitutive high energy mode of growth in the mutant, which would be lethal under conditions where the energy supply is limited. For example, the apparent inability of the double mutant to form chloronemal cells could be lethal in low

light conditions. This is supported by the fact that wild type protonemal tissue grown in low light conditions exclusively consists of chloronemal cells, which helps to meet the increased demands on photosynthetic capacity.

The molecular mechanisms underlying the inability to grow in a normal day/night light cycle has not yet been experimentally addressed in the *snf1a snf1b* double mutant. Functional studies in higher plants have suggested that SnRK1 kinases may be needed to induce genes important for the utilization of stored starch and allocated sucrose (Purcell *et al*., 1998; Laurie *et al*., 2003). In particular, an inability to mobilize starch during the night could potentially explain the inability of the mutant to grow in a normal day-night light cycle.

Conclusions

- The *Arabidopsis* F-box protein Grh1 was cloned as a Mig1 dependent suppressor of *GAL1* gene expression. While the *in vivo* function of AtGrh1 remains to be elucidated, its isolation in this screen confirmed the general feasibility of the "cloning by pathway activation" approach. This method could potentially be used to identify components in other signaling pathways which are conserved between yeast and the organism of interest.

- *PpHXK1* was shown to encode a novel chloroplast stromal hexokinase which is the major glucose phosphorylating enzyme in *Physcomitrella patens*.

- Gene targeting of *PpHXK1* produced an *hxk1* knockout mutant with a complex phenotype affecting growth, development and sensitivities to plant hormones.

- PpHxk1 represents a novel class of plant hexokinases which all have predicted N-terminal chloroplast target peptides. Such hexokinases seem to exist in all plants for which sufficient sequence information is available, suggesting that hexose phosphorylation inside the chloroplasts may be more important than previously realized.

- *PpSNF1a* and *PpSNF1b* encode functionally redundant SnRK1 kinases in *Physcomitrella patens*. Gene targeting produced a *snf1a snf1b* double mutant which was found to be completely devoid of SnRK1 kinase activity.

- The *snf1a snf1b* double mutant exhibits complex developmental phenotypes interpreted by us as a constitutive high energy mode of growth.

- The *snf1a snf1b* double mutant is unable to grow in conditions of low energy supply, which supports the notion that the SnRK1 kinase functions as an energy gauge which is needed to recognize and respond to low energy conditions.

Future perspectives

We intend to continue and broaden our use of *Physcomitrella patens* as a model for plant carbohydrate signaling and regulation of the carbon metabolism. In order to make *Physcomitrella* an even more potent model for studies of plant carbon metabolism, the distribution of carbohydrates on a whole plant level should be investigated. In particular, it would be valuable to know more about carbohydrate storage and to what extent transport of carbohydrates takes place between different cells and tissues.

PpHXK2 encodes a Type B hexokinase in *Physcomitrella*. We would like to elucidate the *in vivo* functions of PpHxk2 and also determine its subcellular localization. This would hopefully shed further light on the functional difference between the two types of hexokinases. In yeast, Hxk2 has been shown to function upstream of the Snf1 kinase in the glucose repression pathway. By creating *snf1a snf1b hxk1* and eventually also *snf1a snf1b hxk2* triple mutants, we will hopefully be able to investigate if such an epistasis exists also in plants.

There remains much to be done regarding the molecular characterization of the *snf1a snf1b* double mutant. SnRK1 from higher plants have been shown to regulate both gene expression and metabolic enzymes (Sugden et al., 1999a; Laurie et al., 2003; Purcell et al., 1998) and this is likely to be the case also in *Physcomitrella*. We are planning to perform a large scale expression analysis where the *snf1a snf1b* double mutant will be compared to the wild type. We will primarily be looking for genes that are normally regulated by energy availability but have lost that regulation in the double mutant. We would also like to use the yeast two hybrid system to screen for *Physcomitrella* proteins which can interact with PpSnf1a and PpSnf1b. The possibility to target the genes encoding such interactors by homologous recombination offers a relatively fast way to subsequently elucidate their functions *in planta*.

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