

# Snf1-related protein kinase 1 is needed for growth in a normal day–night light cycle

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**The yeast Snf1 protein kinase and its animal homologue, the AMP-activated protein kinase, play important roles in metabolic regulation, by serving as energy gauges that turn off energy-consuming processes and mobilize energy reserves during low-energy conditions. The closest homologue of these kinases in plants is Snf1-related protein kinase 1 (SnRK1). We have cloned two SnRK1-encoding genes, *PpSNF1a* and *PpSNF1b*, in the moss *Physcomitrella patens*, where gene function can be studied directly by gene targeting in the haploid gametophyte. A *snf1a snf1b* double knockout mutant is viable, but lacks all Snf1-like protein kinase activity. The mutant has a complex phenotype that includes developmental abnormalities, premature senescence and altered sensitivities to plant hormones. Remarkably, the double knockout mutant also requires continuous light, and is unable to grow in a normal day–night light cycle. This suggests that SnRK1 is needed for metabolic changes that help the plant cope with the dark hours of the night.**

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## Introduction

The ability to monitor the energy status of cells and tissues, and to adjust the metabolism of available nutrients accordingly, is important to all eukaryotes. One example of such regulation is found in the yeast *Saccharomyces cerevisiae*, which preferentially ferments the favoured carbon source glucose. In order to save energy, enzymatic reactions that are needed to metabolize other carbon sources are inhibited in the presence of glucose, mainly by transcriptional repression. This mechanism is known as glucose repression (Ronne, 1995; Johnston, 1999) and affects a large number of genes that are required for the uptake and metabolism of alternative carbon sources as well as genes required for oxidative growth. A key component in glucose repression is the Snf1 protein kinase (Celenza and Carlson, 1986), which

regulates the nucleocytoplasmic localization of the Mig1 repressor (Nehlin and Ronne, 1990) by differential phosphorylation in a glucose-dependent manner (De Vit *et al.*, 1997; Smith *et al.*, 1999).

Genes encoding Snf1-related kinases have been isolated from all eukaryotic kingdoms and it is thought that these kinases share an evolutionary conserved function as metabolic sensors or energy gauges (Hardie *et al.*, 1998). In animals, the kinase is known as the AMP-activated protein kinase, AMPK, since it is regulated by the ATP:AMP ratio. In plants, there are several Snf1-related kinases, which fall into three major subfamilies referred to as SnRK1, SnRK2 and SnRK3 (SnRK: Snf1-related protein kinase) (Halford and Hardie, 1998; Halford *et al.*, 2003, 2004). The SnRK2 and SnRK3 subfamilies are rather divergent in sequence and appear to be unique to plants, while the SnRK1 kinases, based on sequence similarity, are the closest homologues of the yeast Snf1 kinase and the mammalian AMPK.

The biochemical properties of SnRK1 kinases have been extensively studied in several plant species (Halford *et al.*, 2003), and the identification of substrates like HMG-CoA reductase, sucrose phosphate synthase and nitrate reductase (Dale *et al.*, 1995; Sugden *et al.*, 1999) is consistent with a function in metabolic control. This conclusion is also supported by antisense experiments. Thus, Purcell *et al.* (1998) found that potato SnRK1 is needed for sucrose induction of sucrose synthase expression and Zhang *et al.* (2001) showed that barley SnRK1 is required for the formation of functional pollen, possibly because it is needed for starch accumulation. Furthermore, experiments in wheat embryos suggested a role for SnRK1 in regulating the expression of  $\alpha$ -amylase (Laurie *et al.*, 2003). Although many details remain to be elucidated, these findings suggest a role for plant SnRK1 kinases in regulating the carbon and energy metabolism, which is similar to the proposed roles of the yeast Snf1 kinase and the mammalian AMPK (Hardie *et al.*, 1998).

The moss *Physcomitrella patens* has recently emerged as a powerful model system in plant functional genomics following the discovery that gene targeting by one-step gene disruption works in this plant, with frequencies comparable to those in yeast (reviewed by Schaefer, 2002). Furthermore, the main vegetative growth phase of *Physcomitrella* is the haploid gametophyte, which makes it possible to study the phenotype of recessive loss-of-function mutations such as gene knockouts without further crosses. In order to learn more about the function of the Snf1-related kinases in plants, we have cloned two *Physcomitrella* genes, *PpSNF1a* and *PpSNF1b*, encoding SnRK1 kinases and characterized their *in vivo* functions by targeted gene knockouts. Single knockout mutants had no obvious phenotypes except a reduced Snf1-like kinase activity in protonemal tissue extracts. The double knockout mutant is devoid of Snf1-like kinase activity, and has a severe pleiotropic phenotype that includes an inability to grow in a normal day–night light cycle.

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## Results

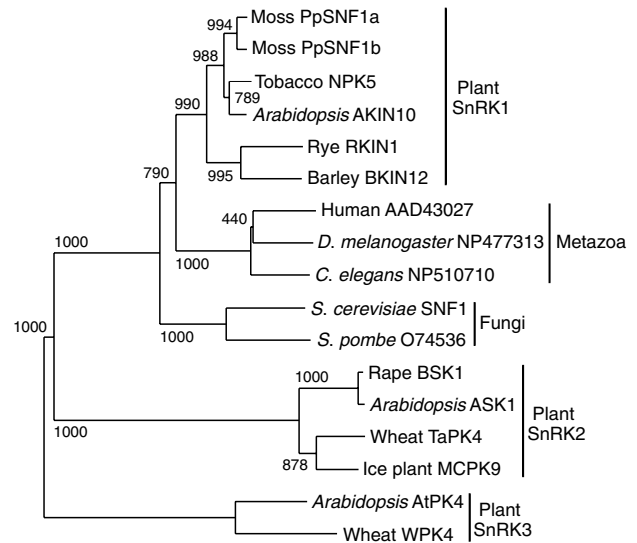
### Two closely related genes encode type 1 Snf1-related kinases in *Physcomitrella*

We used degenerative PCR combined with 5'- and 3'-RACE to clone sequences encoding Snf1-related kinases from the moss *P. patens*. As described in Materials and methods, we obtained full-length cDNAs and the corresponding genomic sequences of two closely related genes that we named *PpSNF1a* and *PpSNF1b*. Both genes have 10 exons that are spliced to form cDNAs encoding highly similar proteins of 542 and 545 amino-acid residues, respectively. The kinase domains (amino acids 19–273) have 96.5% identical residues, while the rest of the two proteins show 67.4% identity to each other. In order to determine if there are any other closely related genes in *Physcomitrella*, we performed low-stringency Southern blots with probes from the kinase domain-encoding regions. These probes are specific for *PpSNF1a* and *PpSNF1b*, respectively, but crosshybridization between the two genes could easily be detected (Supplementary Figure 1). No other hybridizing bands were seen, suggesting that there are no other genes in *Physcomitrella* that are closely related to *PpSNF1a* and *PpSNF1b*.

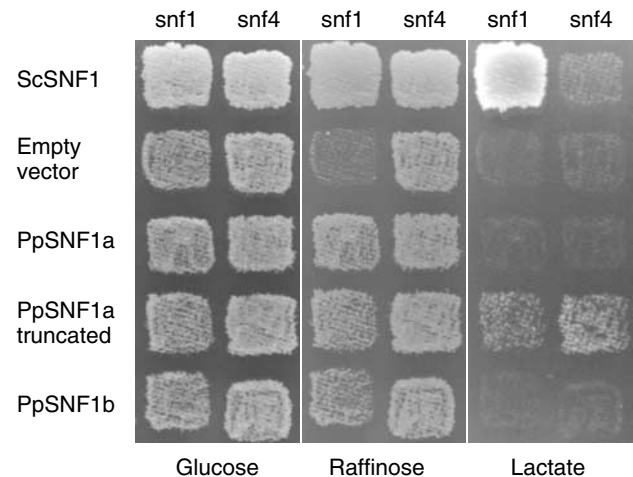
A phylogenetic analysis based on amino-acid sequences clearly shows that *PpSnf1a* and *PpSnf1b* belong to the type 1 (SnRK1) subfamily of the Snf1-related kinases (Figure 1 and Supplementary Figure 2). As expected, they also have an exon–intron organization resembling that of other plant SnRK1-encoding genes, such as *Arabidopsis* AKIN10 (Le Guen *et al*, 1992). We proceeded to examine the expression of *PpSNF1a* and *PpSNF1b*. Since mRNA levels were too low to be easily detected in Northern blots, we used quantitative RT–PCR analysis with internal standards as described by Siebert and Larrick (1992). Total RNA was prepared from young protonemal tissue that had been exposed to different treatments, including growth in the dark or in intense light, and growth in the presence of 0.2 M glucose or mannitol. We found that none of the treatments tested had any significant effect on the expression of either *PpSNF1a* or *PpSNF1b* (Supplementary Figure 3). We conclude that both genes seem to be constitutively expressed in young protonemal tissue.

### *PpSnf1a* and *PpSnf1b* can complement a yeast *snf1* mutant and are regulated by the *Snf4* protein in yeast

Several plant SnRK1 kinases, first exemplified by RKIN1 (Alderson *et al*, 1991), can complement the glucose derepression defect of a yeast *snf1* mutant, thereby enabling it to grow on other carbon sources than glucose. We therefore proceeded to test if the *PpSNF1a* and *PpSNF1b* cDNAs can complement the glucose derepression defects of *snf1* when expressed from the methionine-regulated *MET3* promoter in yeast. The yeast *SNF1* gene expressed from the same promoter was included as a control. As shown in Figure 2, we found that both *PpSNF1a* and *PpSNF1b* can support growth of the *snf1* strain on the non-repressing carbon source raffinose, indicating functional complementation of the *snf1* defect. However, neither cDNA could support growth of the *snf1* strain on lactate, which is a more stringent test for glucose derepression. We conclude that *PpSNF1a* and *PpSNF1b* can partially complement a *snf1* mutant in yeast.



**Figure 1** Phylogenetic tree of Snf1-related kinases showing the positions of *PpSnf1a* and *PpSnf1b*. The conserved kinase domain amino-acid sequences corresponding to residues 20–272 in *PpSnf1a* and *PpSnf1b* were aligned using ClustalX (Thompson *et al*, 1997) with default settings. The tree was computed from the aligned sequences using the neighbour-joining method with correction for multiple substitutions and exclusion of gaps in the alignment. Numbers shown are bootstrap values for branch points based on 1000 samples. The three subfamilies of Snf1-related kinases that have been identified in plants (Halford and Hardie, 1998) are also shown.



**Figure 2** Complementation of yeast *snf1* and *snf4* mutants by the *PpSNF1a* and *PpSNF1b* cDNAs. Yeast strains H316 (*snf1*) and H1307 (*snf4*) were transformed with plasmids expressing the yeast *SNF1* gene, the *PpSNF1a* cDNA, the *PpSNF1b* cDNA, or a truncated *PpSNF1a* cDNA encoding only the kinase domain. The empty vector pJ0177 was also included as a control. Growth was tested on synthetic media containing either glucose, raffinose or lactate, but lacking uracil and methionine in order to select for maintenance of the plasmids and derepression of the *MET3* promoter.

The yeast Snf1 kinase is a heterotrimeric complex made up of the catalytic  $\alpha$  subunit encoded by *SNF1*, a  $\beta$  subunit encoded by the partially redundant *SIP1*, *SIP2* and *GAL83* genes, and a  $\gamma$  subunit encoded by *SNF4* (Jiang and Carlson, 1997). A model has been proposed for how Snf1 is regulated in which a C-terminal regulatory domain binds to the kinase

domain and blocks the active site in the presence of glucose. During glucose depletion, Snf4 binds to the Snf1 regulatory domain, thus relieving this autoinhibition (Johnston, 1999). Consistent with this model, a deletion of the C-terminal regulatory domain makes the yeast Snf1 kinase partly independent of Snf4 (Celenza and Carlson, 1989). We proceeded to test *PpSNF1a* and *PpSNF1b* for their ability to suppress a yeast *snf4* mutant. Since *snf4* mutants can grow on raffinose, growth on lactate was used to score the phenotype (Figure 2). We found that neither *PpSNF1a* nor *PpSNF1b* can suppress the *snf4* phenotype, indicating that they are dependent on Snf4 for their ability to function in yeast. Significantly, we further found that a deletion of the C-terminal domain in *PpSnf1a* made it independent of Snf4, enabling the *snf4* mutant to grow on lactate (Figure 2). We conclude that *PpSnf1a* and *PpSnf1b* not only can complement the kinase activity of Snf1 but are also regulated by Snf4.

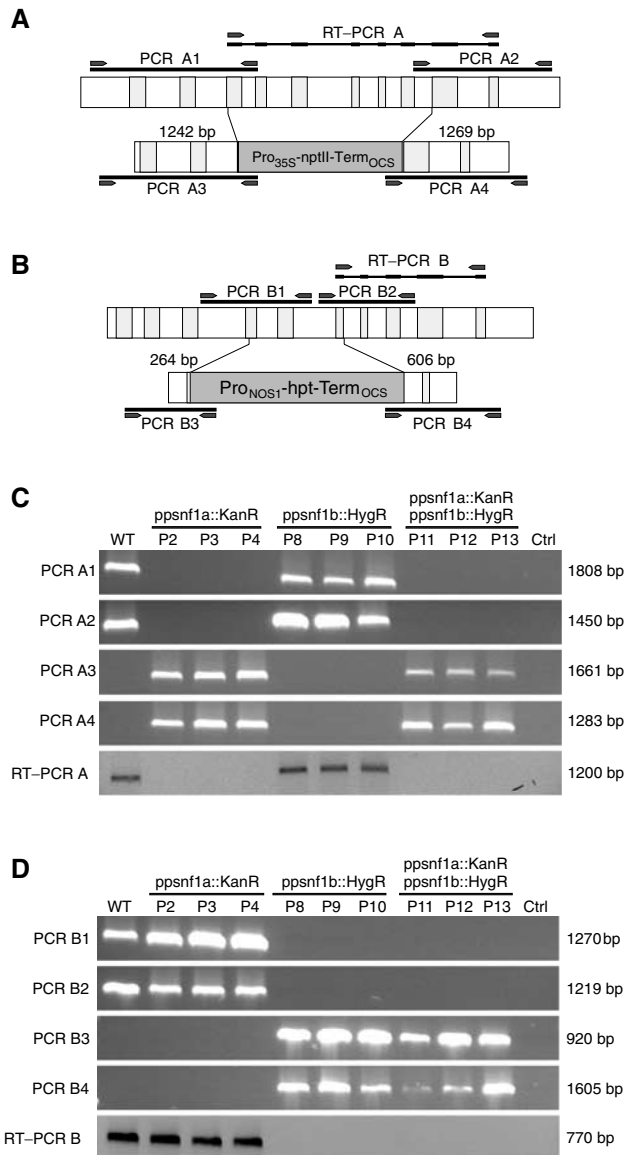
### Targeted disruptions show that *PpSNF1a* and *PpSNF1b* have similar functions *in vivo*

We proceeded to make knockout constructs for both *PpSNF1a* and *PpSNF1b* (Figure 3). To facilitate the generation of double mutants, different selection markers were used in the two constructs: neomycin resistance for *PpSNF1a* and hygromycin resistance for *PpSNF1b*. Moss protoplasts were transformed as described by Schaefer *et al* (1991). Three independent clones from each gene disruption were selected for verification. For each clone, the presence of the disruption in the chromosomal DNA was checked by PCR and the disappearance of the transcript was confirmed by RT-PCR (Figures 3C and D). Initial phenotypic analyses failed to reveal any phenotypes associated with either knockout mutant, except for a partial effect on Snf1-related kinase activity (see below). This suggested that the two genes are functionally redundant, and that a double knockout mutant would be required. Such mutants were generated by transforming a verified *snf1a* mutant with the *PpSNF1b* knockout construct. As with the single knockout mutants, three independent clones were verified (Figures 3C and D) and then further analysed. Unlike the single disruptions, the double knockouts had a number of distinct phenotypes, which are further described below. We conclude that the proteins encoded by *PpSNF1a* and *PpSNF1b* have similar or identical functions *in vivo*. We further note that all clones sharing the same genotype behaved identically under all circumstances tested (for an example, see Figure 5A). Therefore, one representative clone of each knockout, referred to as the *snf1a*, *snf1b* and *snf1a snf1b* mutant, is shown in most of the figures below.

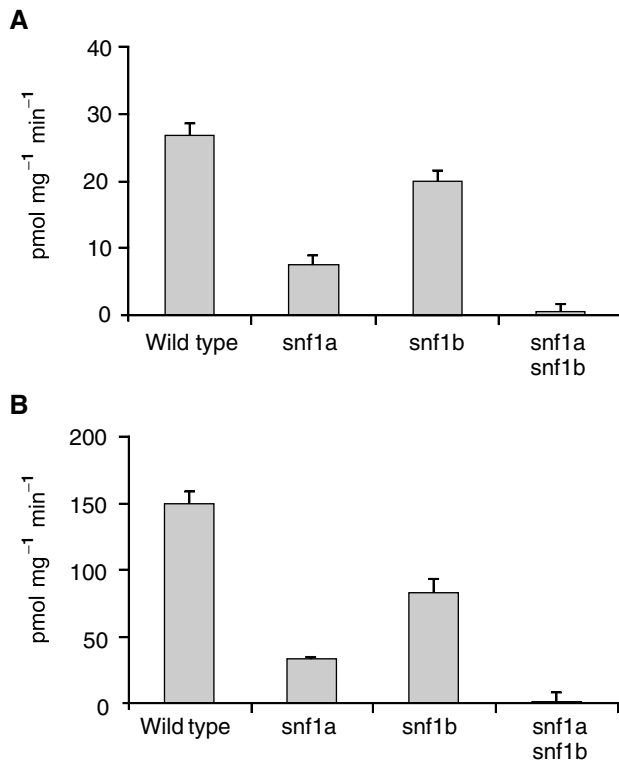
### *PpSnf1a* and *PpSnf1b* together account for all Snf1-like kinase activity in protonemal tissue

Snf1-related kinases from plants, animals and fungi all share similar substrate specificities (Hardie *et al*, 1998) and can therefore be assayed with the same synthetic peptide substrates. A widely used substrate is the SAMS peptide (Davies *et al*, 1989), which is a modified version of the sequence around the AMPK target site in rat acetyl-CoA carboxylase. SAMS-peptide phosphorylating activity in plants was first described by Mackintosh *et al* (1992), and the SAMS peptide has subsequently been used to characterize several Snf1-related enzymes in plants (Halford *et al*, 2003). We proceeded to assay SAMS-peptide phosphorylating activity in crude

extracts from *Physcomitrella* young protonemal tissue (Figure 4A). The assay was linear within the range shown both for total protein added and for time (data not shown).



**Figure 3** Gene targeting of *PpSNF1a* and *PpSNF1b*. (A) Overview of the *PpSNF1a* gene and the *PpSNF1a* targeting construct, in which exons 3–8 were replaced by the G418 resistance cassette. The PCR and RT-PCR products used to verify the deletions in (C) are shown as black bars. The primers used to amplify these products are shown as arrowheads. (B) Overview of the *PpSNF1b* gene and the *PpSNF1b* targeting construct, in which exons 4–6 were replaced by the hygromycin resistance cassette. The PCR and RT-PCR products used to verify deletions in (D) are shown as black bars. The primers used to amplify these products are shown as arrowheads. (C) Verification of *PpSNF1a* knockouts. Genomic DNA was prepared from the wild type (WT) and three independent knockouts of each type (P2–P13) grown on BCD medium under standard conditions. The PCR products A1–A4 were obtained using this DNA as template and the *PpSNF1a*-specific primers shown in (A). RT-PCR products obtained with total RNA prepared from the same strains and *PpSNF1a*-specific primers are shown below. (D) Verification of *PpSNF1b* knockouts. The same strains were used as in (C). The PCR products B1–B4 were obtained using this DNA as template and the *PpSNF1b*-specific primers shown in (B). RT-PCR products obtained with total RNA prepared from the same strains and *PpSNF1b*-specific primers are shown below.



**Figure 4** PpSNF1a and PpSNF1b together account for all SAMS phosphorylating activity in *Physcomitrella*. The enzyme activity is expressed as pmol phosphate incorporated into SAMS peptide per minute and mg of protein. The numbers shown are averages  $\pm$  standard deviations for three samples. For each sample, the activity without added peptide was subtracted from the activity with added peptide. **(A)** Activities in crude extracts of the wild type, the *snf1a* mutant, the *snf1b* mutant and the *snf1a snf1b* double mutant. **(B)** Activities in ammonium sulphate-precipitated fractions (15–30% saturation) of the wild type, the *snf1a* mutant, the *snf1b* mutant and the *snf1a snf1b* double mutant.

We found that protein extracts from wild-type *Physcomitrella* grown under standard conditions have a SAMS phosphorylating activity of  $26.5 \pm 0.9$  pmol mg<sup>-1</sup> min<sup>-1</sup> (Figure 4A). Growth for 20 h in the dark with or without 0.15 M glucose, or treatment with 1  $\mu$ M auxin (IAA) or 1  $\mu$ M cytokinin (BAP) for 20 h did not significantly affect the SAMS phosphorylating activity (Supplementary Figure 4). We conclude that the Snf1-like kinase activity in *Physcomitrella* does not seem to be regulated by these environmental conditions, although it is possible that some *in vivo* regulation exists that is not retained in the extracts.

We next assayed the kinase activity in our knockout mutants. As shown in Figure 4A, both single mutants have significantly reduced activities as compared to the wild type. This shows that both proteins possess Snf1-like kinase activity, with PpSnf1a being the most active enzyme under the conditions tested. Significantly, no activity could be detected in the *snf1a snf1b* double knockout mutant (Figure 4A). This suggests that PpSnf1a and PpSnf1b are the only two Snf1-like kinases in protonemal tissue. This is consistent with the fact that the enzyme activities in the two single knockout mutants add up to the activity in the wild type, indicating that PpSnf1a contributes 72% and PpSnf1b 28% of the total activity. To verify these findings, we proceeded to assay SAMS-peptide phosphorylating activity after partial purification of the ki-

nase by ammonium sulphate precipitation (see Materials and methods). Most of the activity was recovered in the 15–30% ammonium sulphate fraction, where the specific activity had been increased 5.6-fold to 150 pmol mg<sup>-1</sup> min<sup>-1</sup> in the wild type (Figure 4B). This is consistent with results from other plants where Snf1-like kinase activity was recovered in a 0–35% ammonium sulphate fraction resulting in a 3.5-fold purification (Ball *et al*, 1994). The results obtained with the knockout mutants were similar to those described above, with a clearly reduced kinase activity in either single mutant and no detectable activity in the double mutant. Furthermore, the contributions of each kinase as estimated from the single knockout data were identical to those in crude extracts (72% for PpSnf1a and 28% for PpSnf1b). We conclude that PpSnf1a and PpSnf1b together account for all Snf1-like kinase activity in young protonemal tissue.

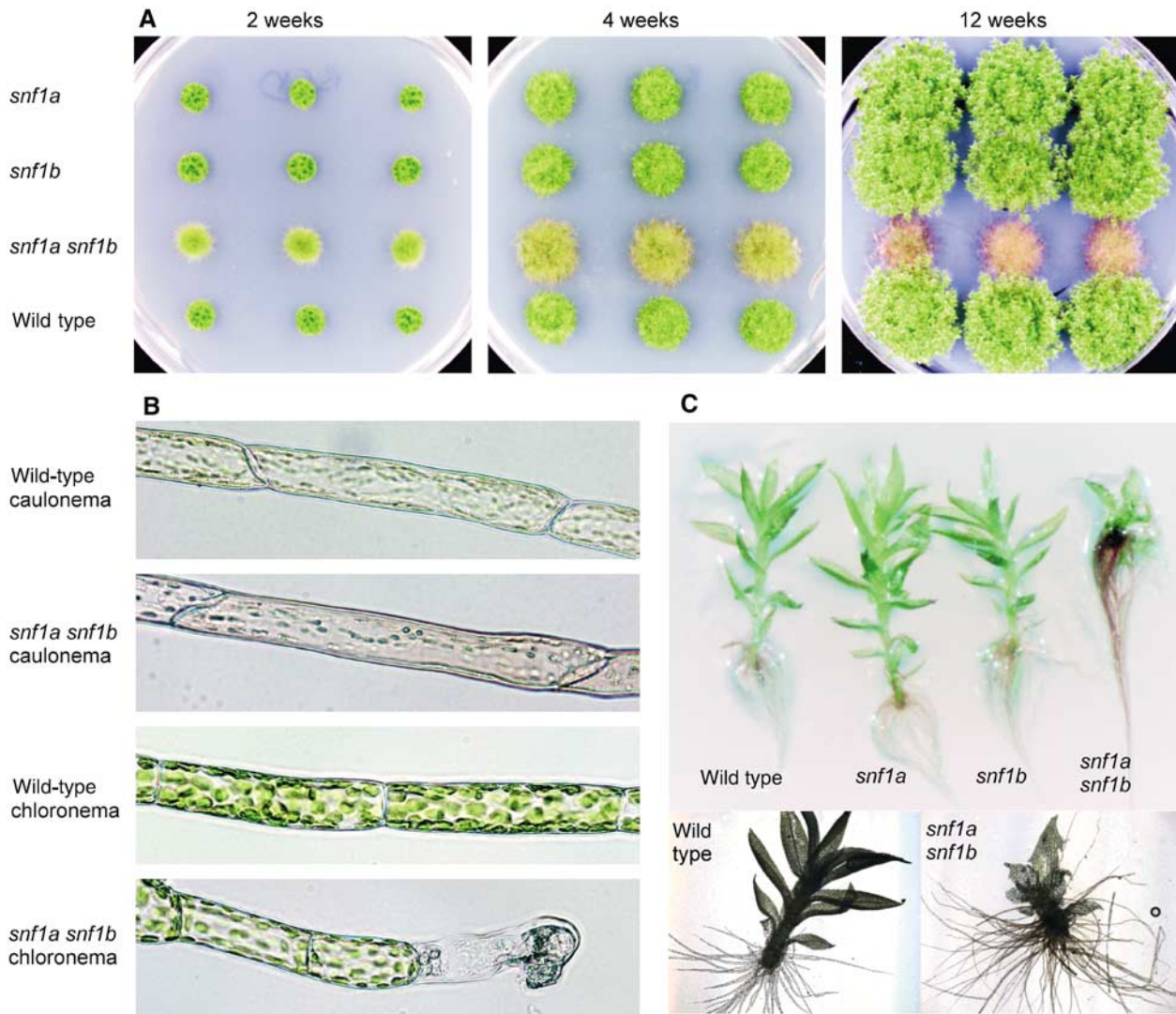
### **The *snf1a snf1b* double mutant has severe pleiotropic phenotypes affecting growth, development and senescence**

The formation of the *Physcomitrella* colony from regenerating spores or protoplasts starts with a filamentous tissue type called protonemata that grow by apical cell divisions (Reski, 1998). There are two types of filaments: chloronemata and caulonemata. The regenerating cells first develop into photosynthetically active chloronemal filaments, which occasionally differentiate into caulonemal cells characterized by oblique cell walls and smaller and fewer chloroplasts. Caulonemal side branch initials can give rise to buds, which subsequently produce gametophores, also known as leafy shoots. Each leafy shoot resembles a small plant with leaves, and has root-like rhizoids at its base.

We found that the *snf1a snf1b* double knockout mutant has several pronounced phenotypes that affect the development of both protonemata and leafy shoots. First, there are striking effects on protonemal filament formation. Thus, while young wild-type colonies consist mainly of a dense network of chloronemal filaments, the *snf1a snf1b* double mutant has a large excess of caulonemal filaments, which grow up into the air (Figures 5A and B). Such caulonemal air filaments are formed sporadically also in the wild type, but in the double mutant they comprise a major part of the colony. Furthermore, those few chloronemal filaments that are formed in the *snf1a snf1b* double mutant are aberrant, with shorter cells and rapid death of the apical cell, particularly under low light conditions (Figure 5B).

Second, there are severe effects on gametophore formation and development in the *snf1a snf1b* double mutant. Very few leafy shoots are formed in the double mutant, as compared to the wild type. Furthermore, those leafy shoots that are formed have shorter stems and smaller leaves with developmental abnormalities ranging from subtle to severe. Interestingly, leafy shoots from the double mutant instead have a large excess of rhizoids (Figure 5C). This suggests that the balance between leaf and rhizoid formation has been disturbed in the double mutant.

A third pronounced phenotype of the *snf1a snf1b* double mutant is its premature senescence. Initially, the double mutant colonies grow faster than wild-type colonies, due to the excess of caulonemal filaments, which promote radial growth. However, the double mutant colonies essentially cease to grow after 4 weeks (Figure 5A). The cells become



**Figure 5** Developmental phenotypes of the *snf1a snf1b* double mutant. (A) Colony morphology of the wild type, the *snf1a* and *snf1b* single mutants, and the *snf1a snf1b* double mutant after 2, 4 and 12 weeks under standard growth conditions (BCD media, 2000 lux, continuous light). Three independent knockout mutants are shown in each case. (B) Morphology of caulonemal air filaments and chloronemal filaments from the wild type and the *snf1a snf1b* double mutant. (C) Leafy shoots from 4-week-old colonies of the wild type, the *snf1a* and *snf1b* single mutants, and the *snf1a snf1b* double mutant.

increasingly pigmented and the colony assumes a wilted appearance. In contrast, the wild-type and the single mutant colonies continue to grow for several weeks under the same conditions with no signs of senescence (Figure 5A).

**The *snf1a snf1b* double mutant is hypersensitive to auxin and hyposensitive to cytokinin**

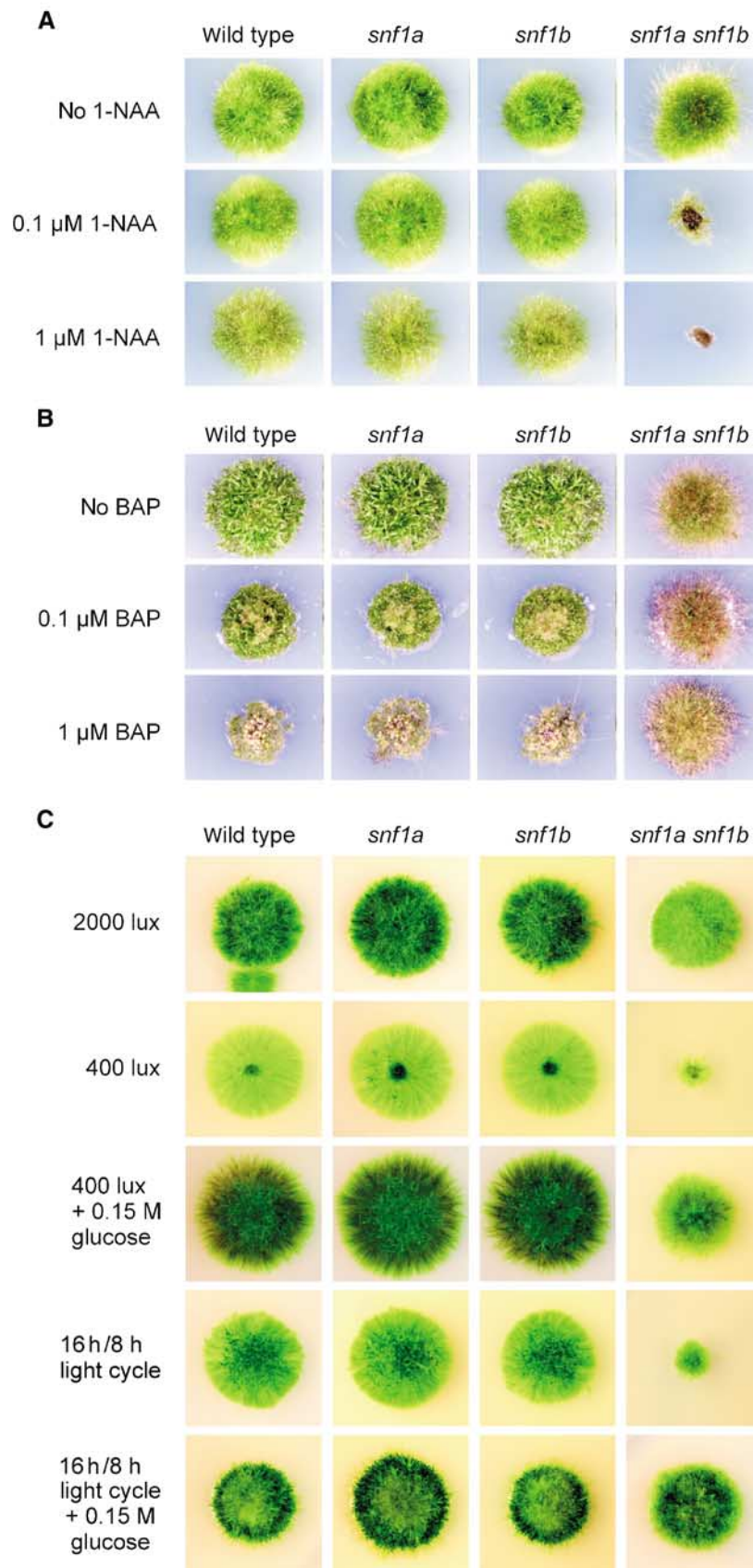
Growth and development in mosses, as in higher plants, is regulated by plant hormones. Specifically, auxins promote caulonema and rhizoid formation in *Physcomitrella* and inhibit the formation of secondary chloronema and leafy shoots, while cytokinins induce bud formation (Ashton *et al*, 1979). We therefore proceeded to examine how our mutants respond to these plant hormones. Wild-type *Physcomitrella* produces colonies with a low-density morphology in the presence of 1  $\mu$ M auxin (Figure 6A), and even higher concentrations inhibit growth (not shown). Interestingly, the *snf1a snf1b* double mutant shows a pro-

nounced hypersensitivity to auxin, with significant growth inhibition at 0.1  $\mu$ M auxin and no growth at all at 1  $\mu$ M auxin (Figure 6A).

Cytokinins at low concentrations induce bud formation in *Physcomitrella*, whereas higher concentrations inhibit growth and promote the formation of calluses (Ashton *et al*, 1979). Consistent with this, we could detect callus formation, accompanied by a reduction in colony size, when the wild-type strain was grown in the presence 0.1  $\mu$ M cytokinin (Figure 6B). In contrast, the *snf1a snf1b* double mutant is hardly affected at all by concentrations up to 1  $\mu$ M cytokinin (Figure 6B). We conclude that the *snf1a snf1b* double mutant is hyposensitive to cytokinin.

**The *snf1a snf1b* double mutant is unable to grow in low light or in a normal day–night light cycle**

Since Snf1-related protein kinases have been proposed to share a common function as gauges of cellular energy levels



**Figure 6** Effects of the *snf1a snf1b* double mutant on growth under different conditions. Small pieces of fresh protonemal tissue were transferred to tester plates on day one. The plates were then incubated under different conditions for 3–4 weeks. In each panel, the wild type and the *snf1a* and *snf1b* single mutants are also shown as controls. (A) Growth in the presence of the auxin 1-naphthaleneacetic acid (1-NAA). (B) Growth in the presence of the cytokinin 6-benzylaminopurine (BAP). (C) Growth in either high or low continuous light, or in a 16 h/8 h day–night light cycle.

(Hardie *et al*, 1998), we proceeded to test how the *snf1a snf1b* double mutant responds to conditions of limited or variable energy supply. As shown in Figure 6C, we found that while the double mutant grows well in high light (2000 lux), it fails to grow at low light intensities (400 lux). Light microscopy analysis of the double mutant tissue grown at 400 lux revealed short chloronemal filaments with a very high incidence of dead apical cells, as previously described in Figure 5B. Significantly, the ability of the double mutant to grow in low light can be partially restored by adding an external carbon source (Figure 6C). This suggests that the failure to grow in low light is not due to a light signalling defect, but instead reflects a failure to adapt to a reduced energy supply.

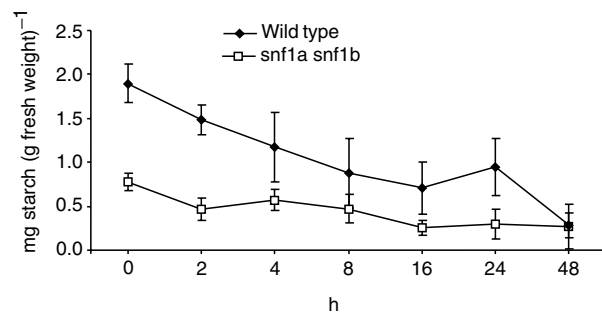
The most obvious example of reduced light conditions that all plants must be able to cope with is the night. We therefore proceeded to examine how the double mutant is affected by growth in a normal (16 h/8 h) day–night light cycle instead of the continuous light used in all preceding experiments. Remarkably, we found that the double mutant is unable to grow in a day–night light cycle, even though high light (2000 lux) is provided for 16 h each day (Figure 6C). Also in this case, growth of the double mutant could be partially restored by the addition of external glucose (Figure 6C). This result shows that the Snf1-related kinase is essential not only for growth in the presence of a fixed but limited energy supply, but also for growth when the energy supply is plentiful but varies during the day. We conclude that the type 1 Snf1-related protein kinases encoded by *PpSNF1a* and *PpSNF1b* are essential for growth in low or variable light, which is consistent with the notion that these kinases are important for handling low-energy conditions.

#### The *snf1a snf1b* double mutant is deficient in starch accumulation

Plants accumulate starch within the chloroplasts during the day, which is subsequently mobilized to serve as an energy source during the night. Experiments in *Arabidopsis* have shown that this transient accumulation and breakdown of starch is important for growth in a day–night light cycle (Caspar *et al*, 1985, 1991; Lin *et al*, 1988), and antisense experiments in wheat have shown that the SnRK1 kinase is important for expression of  $\alpha$ -amylase, a key enzyme in starch mobilization (Laurie *et al*, 2003). The inability of the *snf1a snf1b* double mutant to grow either in the dark or in a 16 h/8 h day–night light cycle therefore suggested that starch metabolism might be affected in the mutant. In order to test this possibility, we assayed the starch content in protonemal tissue from the wild type and the double mutant at different points following a transfer from continuous light to darkness. As shown in Figure 7, we found that the starch content in light-grown double mutant is reduced to 41% of the wild-type value, which suggests that starch accumulation is deficient in the mutant. In contrast, both the wild type and the mutant were able to break down accumulated starch following transfer into the dark, and the difference in starch content disappeared after 48 h in the dark.

## Discussion

The yeast Snf1 kinase and its animal homologue AMPK play key roles in metabolic regulation. Thus, it is thought that



**Figure 7** Reduced starch content in the *snf1a snf1b* double mutant. Protonemal tissue of the wild type and the *snf1a snf1b* double mutant was grown for 9 days in constant high light (2000 lux) and then transferred to darkness. The starch content was quantified after different times in the dark. The error bars show standard errors of mean for six independent samples.

these kinases function as energy gauges that help the cell to adapt to low-energy conditions by turning off energy-consuming processes and mobilizing energy reserves (Hardie *et al*, 1998). In plants, there are several Snf1-related kinases (Halford and Hardie, 1998; Halford *et al*, 2003), but the closest homologue of Snf1 is SnRK1. To learn more about the function of SnRK1 in plants, we have cloned two SnRK1-encoding genes from the moss *P. patens*, where gene function can be studied directly by targeted knockouts in the haploid gametophyte. The two genes, *PpSNF1a* and *PpSNF1b*, encode highly similar proteins (Figure 1 and Supplementary Figure 2), and Southern blots indicate that there are no other closely related genes in the *Physcomitrella* genome (Supplementary Figure 1). *PpSNF1a* and *PpSNF1b* were knocked out by homologous recombination. Since neither single mutant exhibited any obvious phenotypes, we proceeded to make a double knockout mutant.

Significantly, we found that the double knockout mutant is completely devoid of Snf1-like protein kinase activity, as determined using the SAMS-peptide assay (Figure 4). The SAMS peptide is known to be efficiently phosphorylated by SnRK1 kinases from several plants (Halford *et al*, 2003), and our finding therefore suggests that *PpSnf1a* and *PpSnf1b* are the only two SnRK1 kinases that are expressed in *Physcomitrella* protonemata. This is the first time that a null mutant has been reported for Snf1-like kinase activity in any plant or animal. Characterization of the resulting phenotype may therefore shed some light on the role of the Snf1-like kinases not only in plants but also in other eukaryotes. We further note that the SAMS-peptide phosphorylating activity remained unaffected by any of the treatments that we tested, including different light conditions, external carbon sources and exposure to plant hormones (Supplementary Figure 4). This might indicate that the kinase activity is constitutive; however, it is also possible that its *in vivo* regulation is not retained in the extracts. In this context it should be noted that both the yeast Snf1 kinase (Wilson *et al*, 1996) and the mammalian AMPK (Davies *et al*, 1992) require rapid freezing after extraction to preserve the physiological activation state.

The *snf1a snf1b* double mutant exhibits pronounced developmental phenotypes that affect both protonemata and gametophores (Figure 5). In protonemata, there is a clear excess of caulonemal filaments, many of which grow up into

the air. The few chloronemal filaments that are formed in the mutant are aberrant, with shorter cells and a high incidence of dead apical cells. Furthermore, there are fewer gametophores in the mutant, and those that are formed have small malformed leaves and an excess of rhizoids. Growth and development in *Physcomitrella*, as in other plants, is regulated by plant hormones. Thus, auxins promote caulonema and rhizoid formation but inhibit the formation of secondary chloronema and leafy shoots, while cytokinins induce bud formation (Ashton *et al*, 1979). Plant hormone signalling is also known to interact with sugar sensing and signalling, although the mechanisms involved are still poorly understood (Gibson, 2004). Significantly, we found that the *snf1a snf1b* double mutant exhibits altered sensitivities to the plant hormones auxin and cytokinin (Figure 6). We note that these results are consistent with the above-described developmental phenotypes of the double mutant. Thus, its increased sensitivity to auxin could explain the excess of caulonema and rhizoids, and the reduced sensitivity to cytokinin why less buds and leafy shoots are formed. We further note that the altered sensitivities of the double mutant, in particular its auxin hypersensitivity, could reflect a role of the SnRK1 kinase in coordinating growth and development with available energy supplies. According to this interpretation, the kinase would inhibit auxin-dependent signalling under low-energy conditions, thus preventing growth and morphogenesis that the plant cannot afford.

A further pronounced phenotype of the *snf1a snf1b* double mutant is premature senescence (Figure 5A). Snf1/AMPK has been implicated in ageing both in yeast (Ashrafi *et al*, 2000; Lin *et al*, 2003) and in human fibroblasts (Wang *et al*, 2003). However, in these cases, senescence was linked to increased kinase activity, which is the opposite of what we see. Interestingly, results from higher plants have also implicated hexokinase-dependent signalling in senescence (Yoshida, 2003). Thus, overexpression of hexokinase in tomato and *Arabidopsis* promotes senescence (Dai *et al*, 1999; Xiao *et al*, 2000), while an *Arabidopsis* hexokinase mutant instead shows delayed senescence (Moore *et al*, 2003). It should be noted that hexokinase functions upstream of Snf1 in yeast, where it generates a glucose-dependent signal that inhibits Snf1 activity (Rolland *et al*, 2002). Snf1 and hexokinase mutants therefore have opposite phenotypes in yeast. Notably, some of the other phenotypes that we see in the *snf1a snf1b* double mutant, such as increased auxin sensitivity and cytokinin resistance, are also opposite those of hexokinase mutants in *Arabidopsis* (Moore *et al*, 2003). Furthermore, a knockout of the major hexokinase gene in *Physcomitrella*, *PpHXX1*, produces a phenotype that is the opposite of the *snf1a snf1b* phenotype in the sense that less rather than more caulonemal filaments are formed (Olsson *et al*, 2003). Our finding that the *snf1a snf1b* double mutant shows premature senescence is therefore consistent with the notion that the SnRK1 kinases function downstream of hexokinase in plants. This does not necessarily mean that hexokinase functions as a sensor that regulates SnRK1 activity. It is also conceivable that the SnRK1 kinases are regulated by a metabolite downstream of hexose phosphorylation.

A striking phenotype of the *snf1a snf1b* double mutant is its inability to grow in conditions of reduced light (Figure 6C). Thus, it requires continuous high light, and is unable to grow

either in continuous low light or in a normal day–night light cycle where 16 h of high light is followed by 8 h of darkness. Our finding that growth of the double mutant in both cases can be partially restored by the addition of glucose further suggests that it suffers from an inability to handle a reduced or variable energy supply rather than a light signalling defect. A possible interpretation is that the double mutant is locked in a high-energy growth mode, which is lethal when the energy supply is limited or variable. This would be in agreement with the proposed roles of the Snf1-related kinases as energy gauges (Hardie *et al*, 1998). It is also consistent with the excess of caulonemal filaments in the double mutant (Figure 5A), since caulonema formation in *Physcomitrella* requires a sufficient energy supply in the form of high light (Schumaker and Dietrich, 1997).

The inability of the *snf1a snf1b* double mutant to grow in a normal day–night light cycle is particularly intriguing, since an abundant energy supply in the form of high-intensity light is provided for 16 h each day. It is therefore unlikely that lack of energy alone can explain this phenotype. A more likely explanation is an inability to carry out metabolic changes that help the plant cope with the dark hours of the night. A key energy reserve in plants is starch, which accumulates in the chloroplasts during the day, and is mobilized to serve as an energy source during the night. Experiments in *Arabidopsis* have shown that this transient accumulation and breakdown of starch is important for growth in a normal day–night light cycle (Caspar *et al*, 1985, 1991; Lin *et al*, 1988), and several enzymes involved in starch mobilization are preferentially expressed during the night (Harmer *et al*, 2000). Furthermore, experiments in wheat have shown that the SnRK1 kinase is important for expression of  $\alpha$ -amylase, a key enzyme in starch mobilization (Laurie *et al*, 2003). Interestingly, we found that the *snf1a snf1b* double mutant has only 41% of the starch content of the wild type after continuous growth for 9 days in high light (Figure 7). One possible reason for the reduced starch content is the fact that the double mutant has an excess of caulonemal filaments, since the latter contain fewer and less-developed chloroplasts. However, it is also possible that one or more enzymes involved in starch synthesis are regulated by PpSnf1a and PpSnf1b. In this context, we note that fructose-1,6-bisphosphatase, an enzyme needed for starch synthesis, is strictly dependent on Snf1 for its expression in yeast (Hardie *et al*, 1998). Further studies may help to clarify to what extent this or other enzymes involved in starch metabolism are regulated by the two SnRK1 kinases in *Physcomitrella*.

## Materials and methods

### Plant material and growth conditions

The standard growth conditions used were growth at 25°C in a Sanyo MLR-350 light chamber with constant irradiation (2000 lux) from the sides. Protonemal tissue was subcultured on cellophane (#325P, Cannings Packaging Ltd, UK)-covered BCD media (1 mM MgSO<sub>4</sub>, 1.85 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM KNO<sub>3</sub>, 45  $\mu$ M FeSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 1  $\times$  Hoagland's No. 2 solution and 0.8% agar) supplemented with 5 mM ammonium tartrate. For phenotypic analysis, protonemal tissue was precultured for 1 week on cellophane-covered ammonium-free BCD media. Small pieces of fresh protonemal tissue (approximately 2 mm in diameter) were then transferred onto ammonium-free BCD plates without cellophane. The colonies were cultivated for 3 or 4 weeks and then photographed.



### Molecular cloning

Standard methods were used for all molecular cloning (Sambrook *et al*, 1989). The Codehop software (Rose *et al*, 1998) was used to design degenerate PCR primers based on *SNF1*-related plant sequences. Using *P. patens* genomic DNA as template, these primers amplified a 387 bp fragment. The sequence of this fragment was used to design primers for 5'- and 3'-RACE. The resulting RACE products were cloned and sequenced, providing a full-length 2488 bp sequence of the *PpSNF1a* cDNA. *PpSNF1a*-specific primers were then used to amplify a 5089 bp genomic fragment carrying the *PpSNF1a* gene, which was cloned into pCR<sup>®</sup>2.1 to produce pMT103 (Table I). In a low-stringency PCR, *PpSNF1a*-specific primers amplified a faint 648 bp fragment derived from the *PpSNF1b* gene. This fragment was cloned, and its sequence was used to design specific primers for *PpSNF1b*, which were used to obtain overlapping 5'- and 3'-RACE fragments. A unique *Bpu1102I* site within the overlap was used to splice the two fragments into a 2511 bp full-length *PpSNF1b* cDNA. Specific primers were then used to amplify a 5107 bp genomic fragment carrying the *PpSNF1b* gene, which was cloned into pCR<sup>®</sup>2.1 to produce pMT115. The *PpSNF1a* and *PpSNF1b* genomic and cDNA sequences have been deposited in GenBank with accession codes AY347743, AY347744, AY347745 and AY347746. The sequences of the oligonucleotides used will be provided on request.

### Southern blot analysis

*P. patens* genomic DNA prepared from young protonemal tissue was digested to completion with *HindIII*, *BglIII* or *EcoRI*. Using standard blotting procedures (Sambrook *et al*, 1989), the DNA was transferred to nylon filters that were UV crosslinked. The filters were hybridized to <sup>32</sup>P-labelled probes for 4 h at 63°C and were then washed in 0.5 × SSC/0.1% SDS at 63°C. For the *PpSNF1a*-specific probe, we used a 508 bp *NsiI*-*BglIII* cDNA fragment, and for the *PpSNF1b* specific probe, a 526 bp *XmnI*-*MscI* cDNA fragment was used. Hybridizing bands were visualized in a phosphorimager.

### Targeted gene disruptions

To disrupt the *PpSNF1a* gene, a 2446 bp internal *BspEI*-*HpaI* fragment of pMT103 encoding amino-acid residues 131–404 of PpSnf1a was replaced by a 1971 bp selection cassette containing the neomycin phosphotransferase (*nptII*) gene expressed from the 35S promoter followed by the OCS terminator. This selection cassette was excised with *BamHI* and *HindIII* from the plasmid pUBW302. Prior to transformation, the resulting targeting plasmid, pMT104, was linearized with *SmaI* and *XbaI*, thus producing a 4480 bp fragment having 1242 and 1269 bp of *Physcomitrella* DNA flanking the selection cassette on each side (Figure 4A). To disrupt the *PpSNF1b* gene, an internal 1140 bp *AatII*-*BsgI* fragment of pMT115 encoding amino-acid residues 200–323 of PpSnf1b was replaced by a 2578 bp selection cassette containing the hygromycin phosphotransferase (*hpt*) gene expressed from the NOS1 promoter followed by the OCS terminator. This selection cassette was excised with *HindIII*-*XbaI* from the plasmid pMT123. Prior to transformation, the resulting targeting plasmid pMT126 was linearized with *KpnI* and *XmnI* to produce a 3448 bp fragment with 264 and 606 bp of *Physcomitrella* DNA flanking the selection cassette on each side

Table I Plasmids

Plasmid	Insert
pJO177	<i>MET3</i> promoter, 2 $\mu$ origin, <i>URA3</i>
pUBW302	Source of G418 resistance cassette
pMT102	<i>PpSNF1a</i> ORF in pJO177
pMT103	<i>PpSNF1a</i> genomic clone
pMT104	<i>PpSNF1a</i> targeting construct
pMT108	<i>ScSNF1</i> ORF in pJO177
pMT115	<i>PpSNF1b</i> genomic clone
pMT123	Source of hygromycin resistance cassette
pMT126	<i>PpSNF1b</i> targeting construct
pMT139	<i>PpSNF1a</i> RT-PCR internal standard template
pMT140	<i>PpSNF1b</i> RT-PCR internal standard template
pMT141	<i>PpSNF1b</i> ORF in pJO177

(Figure 4B). Moss protoplasts were transformed as described previously (Schaefer *et al*, 1991), and stable transformants were selected in the presence of 50 mg l<sup>-1</sup> geneticin (Sigma G9516) or 30 mg l<sup>-1</sup> hygromycin B (Sigma H3274).

### Complementation in yeast

Yeast media and growth conditions were as described by Sherman *et al* (1986) but with twice as much leucine in the synthetic media. The vector pJO177, which was used for expression of *Physcomitrella* cDNAs in yeast, is a multicopy (2 $\mu$ ) shuttle vector carrying the auxotrophic *URA3* marker and the *MET3* promoter for expression of inserts. A 1764 bp cDNA fragment covering the *PpSNF1a* open reading frame was amplified by PCR and cloned into the *SmaI* site of pJO177 to produce the plasmid pMT102. A 1908 bp *Esp3I*-*HindIII* fragment of the *PpSNF1b* cDNA covering the open reading frame was subcloned into the *SmaI* site of pJO177 to produce plasmid pMT141. The *S. cerevisiae* *SNF1* gene was PCR amplified from yeast genomic DNA and then cloned into the *SmaI* site of pJO177 to produce pMT108. The plasmids were transformed into yeast strains H316 (*MATa snf1::LEU2*) and H1307 (*MATa snf4::LEU2*). Both strains are congenic to W303-1A (Thomas and Rothstein, 1989) and therefore carry the *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* markers as well. Media lacking uracil was used to select transformants. The ability to complement *snf1* knockout mutants was tested on media lacking methionine in order to derepress the *MET3* promoter.

### Protein extractions and kinase assay

Young protonemal tissue grown on cellophane-covered media was used as the starting material for protein extraction. The extraction procedure and subsequent ammonium sulphate fractionation were performed essentially as described by Man *et al* (1997). However, the Roche #1.697.498 proteinase inhibitor mix was used instead of benzamide and PMSF, and PD10 columns (Amersham Biosciences) instead of G50 columns. Moreover, the extraction buffer was changed to fractionation buffer also in the crude extracts that were not subjected to ammonium sulphate precipitation. The SAMS peptide was purchased from Tocris (#1344) and the kinase assay was performed essentially as described (Man *et al*, 1997). We used a SAMS-peptide stock solution of 500  $\mu$ M resulting in a final concentration of 100  $\mu$ M in the assay, and a labelled ATP stock solution of 1 mM ATP with a specific activity of 1650 cpm pmol<sup>-1</sup> resulting in a final concentration of 200  $\mu$ M in the assay.

### Quantitative RT-PCR

Young protonemal tissue grown on cellophane-covered BCD plates for 7 days under standard growth conditions were transferred by disc lifts to tester plates. Conditions tested included growth in the dark or under intense light (6000 lux), and growth in the presence of 0.2 M glucose or 0.2 M mannitol. After 24 h of treatment, the tissue was harvested and total RNA was prepared. Expression levels were measured as the ratio between the transcript-dependent RT-PCR product in 1  $\mu$ g of RNA and an internal standard product. The internal standard was an *in vitro*-transcribed RNA, which was amplified with the same primer pair as the transcript, but producing a differently sized product (Siebert and Larrick, 1992). The primers used recognize stretches within the coding sequences with a limited sequence similarity, and were confirmed not to crossreact between the two transcripts (data not shown). Templates for the *in vitro* transcription of *PpSNF1a* and *PpSNF1b* internal standards were the plasmids pMT139 and pMT140, respectively. The pMT139 insert consists of a *PpSNF1a* genomic fragment deleted for an internal 376 bp *HpaI*/*AflIII* fragment, while pMT140 has an insert consisting of a *PpSNF1b* genomic fragment deleted for an internal 908 bp *BglIII* fragment.

### Starch quantification

Protonemal tissue of the wild type and the *snf1a snf1b* double mutant was grown on BCD plates under standard conditions for 9 days. The plates were then transferred to darkness. Samples were harvested and frozen in liquid nitrogen after 0, 2, 4, 8, 16, 24 and 48 h in the dark. Extraction and quantification of starch were performed using a starch assay kit (Sigma, SA-20). Starch was extracted using the DMSO/HCl method described by the

manufacturer but with 1/4 of the volumes in the protocol. The starch assay was performed according to the manufacturer's protocol but with sample volumes decreased to 1/5 of the original.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

## References

- Alderson A, Sabelli PA, Dickinson JR, Cole D, Richardson M, Kreis M, Shewry PR, Halford NG (1991) Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA. *Proc Natl Acad Sci USA* **88**: 8602–8605
- Ashrafi K, Lin SS, Manchester JK, Gordon JI (2000) Sip2p and its partner *snf1p* kinase affect aging in *S. cerevisiae*. *Genes Dev* **14**: 1872–1885
- Ashton NW, Grimsley NH, Cove DJ (1979) Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta* **144**: 427–435
- Ball KL, Dale S, Weekes J, Hardie DG (1994) Biochemical characterization of two forms of 3-hydroxy-3-methylglutaryl-CoA reductase kinase from cauliflower (*Brassica oleracea*). *Eur J Biochem* **219**: 743–750
- Caspar T, Huber SC, Somerville C (1985) Alterations in growth, photosynthesis, and respiration in a starchless mutant of *Arabidopsis thaliana* (L.) deficient in chloroplast phosphoglucomutase activity. *Plant Physiol* **79**: 11–17
- Caspar T, Lin TP, Kakefuda G, Benbow L, Preiss J, Somerville CR (1991) Mutants of *Arabidopsis* with altered regulation of starch degradation. *Plant Physiol* **95**: 1181–1188
- Celenza JL, Carlson M (1986) A yeast gene that is essential for release from glucose repression encodes a protein kinase. *Science* **233**: 1175–1180
- Celenza JL, Carlson M (1989) Mutational analysis of the *Saccharomyces cerevisiae* SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. *Mol Cell Biol* **9**: 5034–5044
- Dai N, Schaffer A, Petreikov M, Shabak Y, Giller Y, Ratner K, Levine A, Granot D (1999) Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. *Plant Cell* **11**: 1253–1266
- Dale S, Arro M, Becerra B, Morrice NG, Boronat A, Hardie DG, Ferrer A (1995) Bacterial expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-CoA reductase (isoform HMGR1) from *Arabidopsis thaliana*, and its inactivation by phosphorylation at Ser577 by *Brassica oleracea* 3-hydroxy-3-methylglutaryl-CoA reductase kinase. *Eur J Biochem* **233**: 506–513
- Davies SP, Carling D, Hardie DG (1989) Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studies using a specific and sensitive peptide assay. *Eur J Biochem* **186**: 123–128
- Davies SP, Carling D, Munday MR, Hardie DG (1992) Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. *Eur J Biochem* **203**: 615–623
- De Vit MJ, Waddle JA, Johnston M (1997) Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell* **8**: 1603–1618
- Gibson SI (2004) Sugar and phytohormone response pathways: navigating a signaling network. *J Exp Bot* **55**: 253–264
- Halford NG, Hardie DG (1998) SNF1-related protein kinases: global regulators of carbon metabolism in plants? *Plant Mol Biol* **37**: 735–748
- Halford NG, Hey S, Jhurrea D, Laurie S, McKibbin RS, Paul M, Zhang Y (2003) Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. *J Exp Bot* **54**: 467–475
- Halford NG, Hey S, Jhurrea D, Laurie S, McKibbin RS, Zhang Y, Paul M (2004) Highly conserved protein kinases involved in the regulation of carbon and amino acid metabolism. *J Exp Bot* **55**: 35–42
- Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* **67**: 821–855
- Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290**: 2110–2113
- Jiang R, Carlson M (1997) The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Mol Cell Biol* **17**: 2099–2106
- Johnston M (1999) Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. *Trends Genet* **15**: 29–33
- Laurie S, McKibbin RS, Halford NG (2003) Antisense SNF1-related (SnRK1) protein kinase gene represses transient activity of an alpha-amylase (alpha-Amy2) gene promoter in cultured wheat embryos. *J Exp Bot* **54**: 739–747
- Le Guen L, Thomas M, Bianchi M, Halford NG, Kreis M (1992) Structure and expression of a gene from *Arabidopsis thaliana* encoding a protein related to SNF1 protein kinase. *Gene* **120**: 249–254
- Lin SS, Manchester JK, Gordon JI (2003) Sip2, an N-myristoylated beta subunit of Snf1 kinase, regulates aging in *Saccharomyces cerevisiae* by affecting cellular histone kinase activity, recombination at rDNA loci, and silencing. *J Biol Chem* **278**: 13390–13397
- Lin T-P, Caspar T, Somerville C, Preiss J (1988) Isolation and characterization of a starchless mutant of *Arabidopsis thaliana* (L.) Heynh lacking ADPglucose pyrophosphatase activity. *Plant Physiol* **86**: 1131–1135
- Mackintosh RW, Davies SP, Clarke PR, Weekes J, Gillespie JG, Gibb BJ, Hardie DG (1992) Evidence for a protein kinase cascade in higher plants. 3-Hydroxy-3-methylglutaryl-CoA reductase kinase. *Eur J Biochem* **209**: 923–931
- Man AL, Purcell PC, Hannappel U, Halford NG (1997) Potato SNF1-related protein kinase: molecular cloning, expression analysis and peptide kinase activity measurements. *Plant Mol Biol* **34**: 31–43
- Moore B, Zhou L, Rolland F, Hall Q, Cheng W-H, Liu Y-X, Hwang I, Jones T, Sheen J (2003) Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* **300**: 332–336
- Nehlin JO, Ronne H (1990) Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J* **9**: 2891–2898
- 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
- Purcell PC, Smith AM, Halford NG (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. *Plant J* **14**: 195–202
- Reski R (1998) Development, genetics and molecular biology of mosses. *Bot Acta* **111**: 1–15
- Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. *Plant Cell* **14**: S185–S205
- Ronne H (1995) Glucose repression in fungi. *Trends Genet* **11**: 12–17
- Rose TM, Schultz ER, Henikoff JG, Pietrokovski S, McCallum CM, Henikoff S (1998) Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res* **26**: 1628–1635
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

- Schaefer D (2002) A new moss genetics: targeted mutagenesis in *Physcomitrella patens*. *Annu Rev Plant Biol* **53**: 477–501
- Schaefer D, Zryd JP, Knight CD, Cove DJ (1991) Stable transformation of the moss *Physcomitrella patens*. *Mol Gen Genet* **226**: 418–424
- Schumaker KS, Dietrich MA (1997) Programmed changes in form during moss development. *Plant Cell* **9**: 1099–1107
- Sherman F, Fink GR, Hicks JB (1986) *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press
- Siebert PD, Larrick JW (1992) Competitive PCR. *Nature* **359**: 557–558
- Smith FC, Davies SP, Wilson WA, Carling D, Hardie DG (1999) The SNF1 kinase complex from *Saccharomyces cerevisiae* phosphorylates the transcriptional repressor protein Mig1p *in vitro* at four sites within or near regulatory domain 1. *FEBS Lett* **453**: 219–223
- Sugden C, Donaghy PG, Halford NG, Hardie DG (1999) Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase *in vitro*. *Plant Physiol* **120**: 257–274
- Thomas BJ, Rothstein RJ (1989) Elevated recombination rates in transcriptionally active DNA. *Cell* **56**: 619–630
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**: 4876–4882
- Wang W, Yang X, Lopez de Silanes I, Carling D, Gorospe M (2003) Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. *J Biol Chem* **278**: 27016–27023
- Wilson WA, Hawley SA, Hardie DG (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr Biol* **6**: 1426–1434
- Xiao W, Sheen J, Jang JC (2000) The role of hexokinase in plant sugar signal transduction and growth and development. *Plant Mol Biol* **44**: 451–461
- Yoshida S (2003) Molecular regulation of leaf senescence. *Curr Opin Plant Biol* **6**: 79–84
- Zhang Y, Shewry PR, Jones H, Barcelo P, Lazzeri PA, Halford NG (2001) Expression of antisense SnRK1 protein kinase sequence causes abnormal pollen development and male sterility in transgenic barley. *Plant J* **28**: 431–441