Functional Studies of Plant Hexokinases and Development of Genetic Methods in the Moss *Physcomitrella patens*

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2014

Acta Universitatis agriculturae Sueciae
2014:29

ISSN 1652-6880
ISBN (print version) 978-91-576-8006-8
ISBN (electronic version) 978-91-576-8007-5

Print: SLU Service/Repro, Uppsala 2014

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Abstract

In the field of biology, model systems are frequently used for practical reasons. Model organisms possess several features that make them easy to work with in a laboratory setting. In addition they usually have a host of established genetic tools that have been developed by the research community. This thesis explores the potential of adapting methods and tools used in the yeast *Saccharomyces cerevisiae* to the plant model system *Physcomitrella patens*. We have studied the fate and integrity of plasmids transformed into *Physcomitrella* with the purpose of developing a working shuttle plasmids system in moss. Other methods from yeast genetics such as the use of auxotrophic strains would also be useful if adapted to the *Physcomitrella* model system. Experiments using *Physcomitrella patens* showed that a histidine auxotrophic moss strain can be complemented with the wild type gene on a plasmid and that the plasmid can be rescued back into *E. coli*. This indicates that shuttle plasmids, auxotrophic marker genes such as *PpHIS3*, cloning by complementation and perhaps even dosage suppressor screens could be used in *Physcomitrella patens*. Furthermore this thesis investigates the hexose phosphorylating enzyme hexokinase in *Physcomitrella patens* as well as in the charophyte alga *Klebsormidium nitens*. The characterization and localization studies performed on the eleven *Physcomitrella* hexokinases led to the discovery of two new types of plant hexokinases, the type C and type D hexokinases. In addition to these two new types, *Physcomitrella* contained several of the previously described type A and type B hexokinases. In addition to its enzymatic function, hexokinases in both plants and fungi have been implicated in glucose sensing and signaling. This thesis examines if hexokinase 1 of the microalga *Klebsormidium nitens* may play a similar role in glucose sensing as observed in higher plants. With this purpose in mind transgenic lines of the *Arabidopsis thaliana* glucose insensitive mutant *gin2-1* expressing the *Klebsormidium nitens* hexokinase 1 were created. It was found that the *K. nitens* hexokinase can complement the glucose signaling defect in the *gin2-1* mutant.

Keywords: Sugar signaling, hexokinase, *Physcomitrella patens, Klebsormidium nitens*, plant model system, shuttle vector, Auxotroph, HIS knockout

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Dedication

Till min familj, både den lilla och den stora.

Knowing is not enough; we must apply. Willing is not enough; we must do.

Johann Wolfgang von Goethe

None are more hopelessly enslaved than those who falsely believe they are free.

Johann Wolfgang von Goethe

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Murén, E., Nilsson, A., Ulfstedt, M., Johansson, M. & Ronne, H. (2009). Rescue and characterization of episomally replicating DNA from *Physcomitrella*. *Proceedings of the National Academy of Sciences of the United States of America 106(46):19444-9*

II Nilsson, A., Olsson, T., Ulfstedt, M., Thelander, M. & Ronne, H. (2011). Two novel types of plant hexokinases in the moss *Physcomitrella patens*. *BMC Plant Biology 11:32*

III Ulfstedt, M., Hu, G-Z., Eklund, M. & Ronne, H. A charophyte hexokinase can complement both enzymatic and glucose signalling functions in yeast and *Arabidopsis thaliana* hexokinase mutants. (manuscript)

IV Ulfstedt, M., Hu, G-Z. & Ronne, H. Development and testing of an auxotrophic selection marker for use in the moss *Physcomitrella*. (manuscript)

Papers I-II are reproduced with the permission of the publishers.

The contribution of Mikael Ulfstedt to the papers included in this thesis was as follows:

I Took part in the laboratory work by cloning and performing the localisation study for 4 out of 11 hexokinases. Took part in analysing data and made minor contributions to the manuscript.

II Took part in the laboratory work. Work included cultivating moss, PCR experiments and rescuing and characterization of rescued plasmids. Took part in analysing data and made minor contributions to the manuscript.

III Conceived of the project. Performed most of the planning and most of the laboratory work. Wrote the initial draft of the manuscript.

IV Took part in the project conception. Performed most of the planning and most of the laboratory work. Made large contributions to the manuscript.

Abbreviations

ABA	Abscisic acid
AMPK	AMP-activated kinase
ATP	Adenosine triphosphate
At	Arabidopsis thaliana
bZIP	Basic leucine Zipper
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
GFP	Green fluorescent protein
HR	Homologous recombination
HXK	Hexokinase
Kn	Klebsormidium nitens
NHEJ	Non-homologous end-joining
NHR	Non-homologous recombination
ORF	Open reading frame
PCD	Programmed cell death
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pp	Physcomitrella patens
RNA	Ribonucleic acid
SNF1	Sucrose nonfermenting-1
SnRK	Snf1-related kinase
T6P	Trehalose-6-phosphate
TOR	Target of Rapamycin

1 Introduction

If one were to view our home the planet Earth from space, one would observe the rich blue of the oceans, the white of clouds and ice caps and the earthy colours of land. Looking more closely one would also see patches of green. This green colour visible from space is of course plants. The green plants are the primary producers of planet Earth, harvesting the energy of the sun and storing it in chemical bonds. Through the process of photosynthesis plants synthesize carbohydrates from carbon dioxide and water. The vast majority of all life on the planet is dependent on this process for their survival. Green plants also provide most of the world's molecular oxygen as a byproduct of photosynthesis. Also important to the survival of different organisms, be they small single celled organisms like the yeast *Saccharomyces cerevisiae* or larger multi-cellular organism like the bryophyte *Physcomitrella patens*, is the ability to sense if there are available nutrients that can sustain their growth and development. By using model systems like the two species mentioned above one can study different processes that occur within these organisms and hopefully gain a greater understanding of all living things.

The work described in this thesis uses model systems to characterize and study one specific intra-cellular protein, an enzyme known as hexokinase. Hexokinases play a key role in carbohydrate metabolism and also help to sense the availability of glucose in order to regulate the metabolism of the cell.

The model systems used for biological studies are only as good as the tools one can use in said systems, with this principle in mind we in addition to the study of hexokinases have also sought to adapt methods from molecular yeast genetics for use in the moss *Physcomitrella patens*. It would be of great value to plant research if some of the powerful tools used in yeast genetics could be made to work in plants.

This thesis summary contains a brief overview of plant carbon and energy metabolism and then looks more closely at what is known about hexokinases

and glucose signaling. It discusses the hexokinases of *Physcomitrella patens* their characteristics and subcellular localization in more detail. It moves on to discuss the single hexokinase found in the microalga *Klebsormidium nitens* and what it can tell us of the evolution and adaptation of plant hexokinases.

The thesis also gives a summary of the *Physcomitrella patens* plant model system and its efficient native system for homologous recombination that enables gene targeting and the creation of knockouts strains. We have studied the fate of plasmids transformed in to moss with the aim of developing a functional shuttle vector system for *Physcomitrella.* We also show how the use of auxotrophic strains as seen in *Saccharomyces cerevisiae* has the potential to be adapted for use in *Physcomitrella patens* hopefully expanding the utility of *Physcomitrella* as a model system.

2 Model organisms

In the early history of our planet, organisms evolved who were able to form energy rich molecules out of simple inorganic substances. The most successful of these autotrophs were the early ancestors of plants. These primitive autotrophs lived and evolved in the oceans over 3 billion years ago. Life would remain in the sea for a very long time. Approximately a billion years ago multicellular life evolved and around 600 million years ago plants conquered land when certain types of algae adapted to life on land (McCourt *et al.*, 2004). The common ancestor to all land plants belonged to the charophytes, a group of fresh water algae.

Some of the first plants to evolve on land were the liverworts, hornworts and bryophytes and these primitive land dwelling plants have survived to the modern age. Bryophytes are generally referred to as mosses, and are thought to have separated from vascular plants around 450 million years ago (Rensing *et al.* 2008). Still, bryophytes contain most of the important adaptive changes that terrestrial plants developed in order to survive on land.

The world as we know it is full of diverse multitude of living organisms, and there is probably in the excess of 300 000 different plant species (Current results, 2014). In order to study certain organisms in more detail the scientific community has generally chosen to work with model organisms that allow a more in depth look and greater knowledge of the specific model systems. The assumption is that what holds true for a certain model system is also largely true for other similar organisms. Certain organisms are better suited to be model organisms than others. Some features that make attractive qualities for model systems are for example that they are easy to maintain in a laboratory setting, readily reproduce and have short generation times. It is also good if the organism easily lends itself to methods of genetic manipulation and has a genome that has been sequenced.

2.1 *Physcomitrella patens*

2.1.1 The moss *Physcomitrella patens*

The bryophyte *Physcomitrella patens* is a plant that is well suited as a model system for higher plants. Even though bryophytes diverged from seed plants more than 400 million years ago they still possess many of the structures found in higher plants. They mostly respond to changes in their environment and to plant hormones in similar ways as higher plants do (Cove 2005; Cove *et al.*, 2006; Nishiyama *et al.*, 2003). One exception is gibberellin (GA) signaling which appears to be absent in moss (Hirano *et al.*, 2007).

Even though *Physcomitrella* has many similarities to higher plants, it is a much simpler organism to work with. *Physcomitrella* is small and very easy to cultivate, and it grows readily on agar plates, thus eliminating the need for large green-house facilities. This also makes it easy to grow *Physcomitrella* under defined and controlled conditions. The moss is also very easy to propagate since a new moss colony can be regenerated from a single cell taken from any part of the plant.

Furthermore, *Physcomitrella* is a very attractive model system for genetic and developmental studies. The haploid phase of the gametophyte is the dominant phase in the life cycle (Engel, 1968). This makes it possible to observe recessive traits directly without having to do time consuming backcrosses in order to obtain homozygous plants.

Physcomitrella has been used for genetic studies for more than 50 years, but it is only within the last two decades that the use of *Physcomitrella patens* as a model system has really taken off. The catalyst for this was the discovery that *Physcomitrella* has an efficient native system of homologous recombination (Schaefer & Zrÿd, 1997). This opened up the possibility for extensive genetic studies in *Physcomitrella patens* using gene targeting and allele modifications. A number of genetic tools have been adapted from other model systems to be used in *Physcomitrella*, like RNAi interference (Bezanilla *et al*., 2003). In addition to these tools, the genome of *Physcomitrella patens* was the first non-seed plant genome that was completely sequenced and annotated (Rensing *et al.*, 2008).

2.1.2 The life cycle of *Physcomitrella patens*

The moss *Physcomitrella patens* together with ferns and seed plants has alternation of generations between a haploid and a diploid phase (Figure 1). But unlike seed plants, where the diploid phase is the dominant one, the haploid phase is dominant in *Physcomitrella*. The haploid phase constitutes most of what we recognize as the moss plant. The haploid spore germinates by

producing long filaments, these filaments are called chloronemal filaments and is one of two types of protonemal filaments, the other being the caulonemal filaments. Chloronemal cells are recognized by their perpendicular cell walls and the presence of large and well developed chloroplasts within the cells. Caulonemal filaments have oblique cell walls, fewer and less developed plastids, and longer cells (Reski, 1998). The chloronemal filaments that grow out from the spore will eventually give rise to caulonemal filaments, this differentiation is induced by the plant hormone auxin (Cove, 2005). Side branches from chloronemal filaments only form other chloronemal filaments whereas side branches formed from the caulonemal filaments can consist of caulonemal cells or differentiate back into chloronemal cells. On the caulonemal filaments, buds may eventually form, which will develop into leafy shoots called gametophores that resemble the diploid life stage of higher plants. Gametophores consist of a stem-like structure with leaves along the axis of this stem. These leaves are only one cell layer thick and consist primarily of cells that resemble the cells of the chloronemal filaments, but the cells at the outer edge of the leaf instead resemble caulonemal cells. At the base of the gametophores are the rhizoids, long filaments resembling roots that provide support to the gametophores, thus helping the gametophores keep themselves upright. The gametophores do not have a vascular system as seen in higher plants, but *Physcomitrella* has certain conductive cell strands that could be used to transport fluids (hydroids) and also nutrient conductive cells (deuters) (Sakakibara *et al.*, 2003). The gametophore is also were the gametangia are located. These develop at the apex of the gametophores in response to a decrease in temperature and a shorter light cycle, in other words conditions that are seen in the autumn. Both the female sexual organs, called archegonia, and the male sexual organs, called antheridia, develop on the same gametophore. Fertilization requires a small amount of water so that the spermatozoids produced in the antheridia can swim using their flagella and thus reach the egg in the archegonia. The resulting zygote develops into the sporophyte. The diploid sporophyte sits on top of the gametophore, and once fully developed it consists of a spore capsule and a short stem referred to as seta. Inside the sporophyte meiosis takes place and produces roughly 4000 haploid spores (Cove, 2005). When the spore capsule later dries it will break and release the spores. Each spore is capable of starting a new moss colony after germinating.

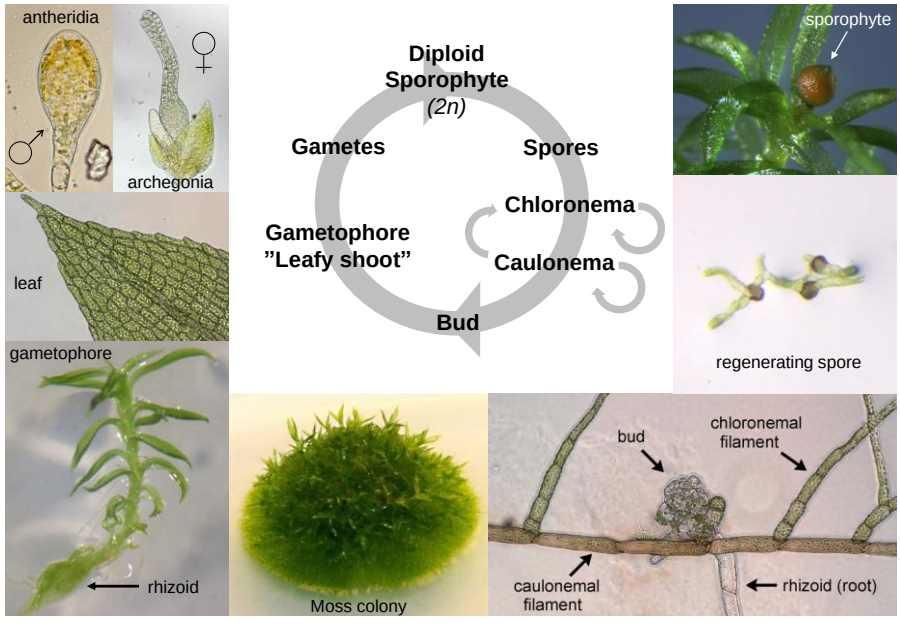

Figure 1. Life cycle of *Physcomitrella patens*. Photos by Anders Nilsson and Mattias Thelander

2.1.3 The *Physcomitrella* genome

The *Physcomitrella patens* genome has been sequenced and assembled into 480 Mbp of scaffolded sequences with an EST coverage of over 98% (Rensing *et al.*, 2007). The general consensus on the number of chromosomes is 27 which is more than in most moss species. As is common with mosses the chromosomes tend to be small (Cove, 2005). Knowing the exact number of chromosomes is difficult, since many mosses also have small micro-chromosomes. These micro-chromosomes are most likely nonessential B-chromosomes (Dyer & Duckett, 1984). The assembled genomic sequence contains 35,938 predicted and annotated gene models. 84% of the predicted proteins appear to be complete and about 20% of the genes show alternative splicing, which is similar to findings in other plants such as rice and *Arabidopsis*. Evidence suggests that *Physcomitrella patens* has gone through at least one whole genome duplication (Rensing *et al.*, 2007). In a study where transcripts were analyzed with respect to their biological function, it was observed that 70-80% of them were involved in the metabolism, which is much more than the fraction in seed plants, where between 10% and 44% is observed (Lang *et al.*, 2005). When the complete nuclear genome sequence was analyzed it was confirmed that *Physcomitrella* has more genes involved in the

metabolism (Rensing *et al.*, 2007). It has therefore been suggested that *Physcomitrella* has metabolic pathways that are not found in higher plants (Nishiyama *et al.*, 2003).

2.2 *Arabidopsis thaliana*

Arabidopsis thaliana is a small flowering plant of the mustard family (Brassicaceae). It is an annual plant native to Europe and Asia that holds no commercial value but has become the major model system used by the plant research community as measured by the number of publications (Koornneef & Meinke, 2010).

Arabidopsis has several features that make it a good model system. Its small size means that it can be maintained within a relatively small growth facility, and it also has a short generation time of about 8 weeks which facilitates genetic studies. It reproduces by self-pollination and gives a good yield of seeds, usually thousands of seeds from a single individual. All this is helpful because often it is desirable to produce as many offspring from a single *Arabidopsis* mutant as possible. Although the plant self-pollinates it is also possible to do cross pollination to create offspring from two different parental lines.

Many genetic and genomic tools have been developed for *Arabidopsis*, this has been of great help to the *Arabidopsis* research community and has advanced the research on not just *Arabidopsis* but plants in general. Perhaps the most important method developed is the ability to transform and genetically alter the *Arabidopsis* plant. The fact that *Arabidopsis* is the most well studied flowering plant led to an early push to sequence the *Arabidopsis* genome, work that was completed in 2000 (Somerville & Koornnee, 2002). The genome contains 5 chromosomes with a total of 125 Mbp (Kaul *et al*. 2000). It is one of the smallest genomes among the flowering plants. There are also large collections of *Arabidopsis* lines that are transgenic or carry mutations. These mutant *Arabidopsis* plants have been used to study many different processes in plants and are freely available to researchers.

2.3 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae commonly known as budding yeast or baker's yeast is the quintessential model system. *Saccharomyces* has a long history of being appreciated by humans for its role in brewery and baking, but as one of the simplest eukaryotic organism it has several great properties as a model system as well. Yeast is a single cell haploid microorganism but it still possesses many

of the same cellular processes as found in other eukaryotic cells like those of plants and animals. Thus, from a biological standpoint what applies to yeast cells is often also true for plant and animal cells. Many human disease genes that involve basic cellular mechanisms like cell division and control of gene expression have homologues in yeast. The fully sequenced yeast genome consists of 12 million base pairs divided on 16 chromosomes that harbor some 6000 genes. This small genome together with the efficient native system for homologous recombination that enables exceptionally efficient gene targeting makes yeast a very attractive model system. In addition to being easy as well as fast to cultivate, there are also extensive knockout strain collections available that cover the entire genome. The main limitation of yeast as a model for higher eukaryotes is the fact that many genes and processes found in higher eukaryotes do not exist in yeast and therefore cannot be studied using this model organism.

2.4 *Klebsormidium nitens*

Klebsormidium nitens is not yet an established model system but this thesis deals in part with this organism, so it deserves to be mentioned. As pointed out above the common ancestor of all land plants belonged to the charophyte algae. Charophytes range from large multicellular species to small unicellular microalgae. One of these charophyte microalgae is *Klebsormidium nitens*, a filamentous uniseriate alga that is characterized by cells having a parietal chloroplast. *Klebsormidium* is a widespread taxa of microalgae observed in polar as well as in tropical regions. It can be found in both terrestrial as well as freshwater habitats, in rivers and bogs and on exposed surfaces of rocks and trees. As a group of organisms that stand between other algae and land plants, the charophytes could offer unique insight into the adaptive changes that occurred when water living plants adapted to life on land.

3 Molecular genetics in *Physcomitrella patens* and yeast

3.1 DNA repair and homologous recombination

Mutation in the genomic DNA is something that frequently occurs, but while mutations are necessary for natural selection and indeed responsible for the evolution of all species past and present, most mutations are deleterious. Deleterious mutations by definition cause harm to the cell or individual that carries them. For this reason systems for DNA repair have evolved within the cell. Mutations in the form of single-stranded DNA lesions and point mutations can for instance be repaired by nucleotide excision repair. This process involves the excision of 20-30 base pairs of the single-stranded sequence containing the mutation. This is followed by repairing the gap by the synthesis of new DNA using the intact single-stranded DNA as a template (Kimura & Sakaguchi, 2006).

Another form of DNA damage that is much more dramatic is double-stranded DNA breaks. This type of damage can be particularly dangerous to the organism because it may result in a permanent loss of genetic material. Since there is no opposing strand to use as template as in the case of nucleotide excision repair, the cell must use other means to repair the DNA. Double stranded breaks can result from environmental damage such as energy rich radiation but can also be induced by the organism itself, for instance during meiotic recombination. While the mechanisms involved in repair of double stranded breaks and the cellular machinery used is not fully understood it appears that DNA breaks can be repaired in two ways, by homologous recombination (HR) and by non-homologous recombination (NHR). The HR pathway relies on a homologous sequence located on another DNA molecule to repair the double stranded break (Figure 2). HR can also be used for artificial manipulation of the genome; it is the mechanism by which gene targeting

works in model systems like yeast (Figure 2a) and *Physcomitrella* (Figure 2c). The second method, non-homologous recombination, fuses sheared DNA ends with little or no regard to their overall homology. NHR can further be divided into non-homologous end joining (NHEJ) (Figure 2b and Figure 2d). and micro-homology-mediated end joining (MMEJ). In NHEJ, sheared DNA ends that are in close proximity are processed to create short overlapping sequences or blunt ends that are subsequently ligated. MMEJ requires that the DNA ends are processed to create single stranded DNA that is able to pair with each other through micro-homologies. Both types of NHR often result in DNA insertions or deletions significantly altering the DNA sequence. Whether double-stranded breaks are repaired through NHR or HR may depend on the phase in the cell cycle. In G_2, each chromatid has a sister chromatid, making HR an appropriate way of repairing broken DNA molecules (Kamisugi & Cuming, 2009).

The native system of homologous recombination present in yeast and *Physcomitrella* is remarkably efficient, something which makes *Physcomitrella* unique among multicellular eukaryotes (Schaefer & Zrÿd, 1997). A possible reason for this efficient HR is that haploid organisms such as yeast and *Physcomitrella* are more vulnerable to double stranded breaks since they lack the redundancy of a diploid organism. It is reasonable to speculate that the efficient HR seen in these two organisms might be a way to compensate for this. The exact mechanism responsible for homologous recombination in *Physcomitrella patens* is still unclear, but it involves the MRN complex consisting of proteins like PpMRE11, PpRAD50 and PpNBS1. It was recently found that PpMRE11 and PpRAD50 are essential for gene targeting in *Physcomitrella* but PpNBS1 is not (Kamisugi *et al.*, 2012). These proteins bind to the double-stranded breaks and produce single stranded 3' overhangs that are able to anneal to a homologous sequence. If there are any non-homologous sequences at the end of a single stranded DNA with homology elsewhere, these non-homologous ends will be removed. This mechanism could explain the formation of concatemers in moss (see below) as well as the deletions seen due to direct repeat recombination within plasmids (**I**). In animal cells and among filamentous fungi, RAD51, the eukaryotic homologue of the bacterial RecA, is required for gene targeting, whereas the protein RAD52 plays a minor role. In contrast, RAD52 in *Saccharomyces cerevisiae* is essential for gene targeting whereas RAD51 plays only a supporting role. In *Physcomitrella* it has been shown that the loss of the two partially redundant RAD51 genes eliminates gene targeting and increases the rate of illegitimate recombination (Schaefer *et al.*, 2010). No RAD52 orthologues have been found in plants. The processes for homologous recombination is without a doubt complex and involves other genes than those mentioned above, one study shows that the PpMSH2 gene

also plays a role in homologous recombination. Knockouts of this gene consequently reduce the efficiency of recombination between homologous sequences. It also affects recombination between homologous but non-identical sequences. Sequences that differ up to 3% from a defined allele sequence are just as efficiently targeted in *Physcomitrella* as identical sequences (Trouiller *et al.*, 2006).

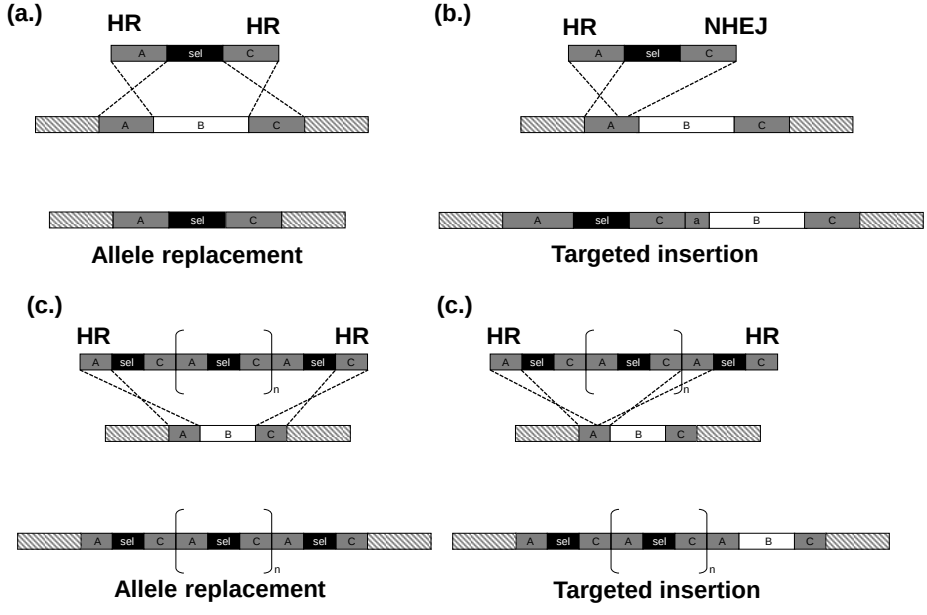

Figure 2. Targeted gene replacement and targeted insertions using homologous recombination. **a**, allele replacement with one linear plasmid. **b**, targeted insertion with one linear plasmid. **c**, allele replacement with a concatemer of several fused plasmids. **d**, targeted insertion with a concatemer of several fused plasmids

3.2 Homologous recombination and gene targeting in *Physcomitrella patens*

The moss *Physcomitrella patens* exhibits a high frequency of targeted gene replacement, similar to that of the budding yeast *Saccharomyces cerevisiae*, when transformed with DNA constructs containing sequences homologous to the moss genome. This makes *Physcomitrella* an attractive system for

functional genetic studies, and has established *Physcomitrella* as a model plant. The unusually high frequency of homologous recombination in *P. patens* is from what is known unique among multicellular organisms. It appears that gene targeting and replacement also works in other mosses such as *Ceratodon purpureus*, but the gene targeting in *Ceratodon* is not as efficient as in *Physcomitrella* (Brücker *et al.*, 2005).

The length of the homologies has direct bearing on the efficiency of recombination. It has been shown that gene targeting works at a frequency of 50% when the homologous flanking sequences are around 600 bp in length. The symmetry of the targeting construct is also important. If one homologous flanking sequence is substantially longer than the other, the construct will tend to integrate into the genome with one single homologous recombination event at the long end and a non-homologous end joining at the short end. Non-homologous end joining often results in unwanted rearrangements at the site of the integration (Kamisugi *et al.*, 2005). An additional factor that can affect the frequency of allele replacement is where in the genome the construct is targeted to, since certain regions seem more difficult to target than others. It also seems that having flanking regions on the construct where the homologous sequences go all the way to the end helps targeting, perhaps because homologous invading DNA ends increase the efficiency of the process (Kamisugi *et al.*, 2005). One thing about the *Physcomitrella patens* system for homologous recombination that is fundamentally different from that in *Saccharomyces cerevisiae* is that transformed DNA constructs tend to form concatemers *in planta* and then integrate at the target locus as an array with multiple copies of the construct. If the moss is transformed with a single type of DNA construct (Figure 2c and 2d), these concatemers tend to arrange in a tandem head-to-tail configuration. If transformation is done with multiple constructs, the resulting concatemers are formed with the individual constructs in all possible orientations (Kamisugi *et al.*, 2006). Our study into plasmids in moss (**I**) indicates that the formation of these concatemers may be due to both precise ligation of linearized plasmids and due to recombination between homologous sequences.

3.3 Extrachromosomal transgenic elements in *Physcomitrella patens*

Foreign DNA that is transformed into *Physcomitrella patens* can also replicate episomally, without integrating into the genome. Episomally replicating DNA in the moss will usually be lost quite rapidly if there is no selection for it. On the other hand, it has been shown that a moss strain transformed with a plasmid

carrying a selective marker can retain the plasmid for more than 10 years as long as it is subcultured on selective media, but if the same moss strain is moved to and subcultured on nonselective media, it will quickly lose the plasmid (Ashton *et al.*, 2000).

Transforming moss with the purpose of transiently expressing a gene on a plasmid is something that works well and is widely used for research purposes. Generally three classes of transformants can be obtained when moss protoplasts are transformed with a construct containing a selective marker (Schaefer, 1994):

Class I
Class I transformants are obtained at a frequency of 10^{-4} to 10^{-5}. These transformants are chromosomally integrated, and as mentioned above this process is stimulated by having sequences homologous to the moss genome on the plasmid.

Class II
Class II is the most common type of transformants with a frequency of about 10^{-2}. They represent episomaly replicating DNA. Class II transformants are unstable and lose the transformed DNA at a high frequency, and this type of transformants therefore grow poorly under selection.

Class III
The third class of transformants are rare, with a frequency of less than 10^{-5} and are believed to represent stable episomally replicating DNA. In contrast to the class II transformant, they are not easily lost, and the transformants therefore grows well under selection.

It is unclear what the biological differences are between the Class II and Class III transformants. It has been suggested that it could involve the incorporation of moss DNA into the class III plasmid/concatemers and that this moss DNA would somehow stabilize the replication or segregation of the transformed DNA (Schaefer, 1994). It was also suggested that yeast artificial chromosomes (YAC vectors) may be more stable in *Physcomitrella patens* (Schaefer, 1994).

3.4 Shuttle vectors and suppressor screens

In yeast, shuttle vectors have a long history and have proved to be a very useful tool for researchers. What signifies a shuttle vectors, usually a plasmid, is that it can propagate in two different species. Within yeast research the other

organism in which the plasmid can replicate has almost always been *E. coli*. The reason for this is that plasmids in *E. coli* can more easily be replicated, manipulated and studied. This means that the shuttle plasmid needs to contain a bacterial origin of replication as well as a yeast origin of replication in addition to some type of selectable marker for each organism. In *E. coli* this is usually a gene enabling resistance to a specific antibiotic, in yeast it is often a marker gene complementing a metabolic pathway defect in an autotrophic yeast strain. Yeast shuttle plasmids can either use chromosomal origins of replication, termed autonomously replicating sequences (ARS) or the 2µ origin. The 2µ origin originally comes from the endogenous 2µ plasmid found in yeast (Beggs 1978; Stinchcomb *et al.*, 1979).

Plasmids containing ARS origins are very unstable; they tend to be present in variable copy numbers and are frequently lost due to failure to segregate to the daughter cell. Plasmid with the 2µ origin on the other hand will usually propagate better, have a higher copy number and be more stable. The stability of ARS plasmids can be improved by adding a yeast centromere (CEN) to the plasmid, which will help the plasmid to segregate to the daughter cell using the cell's own cell division machinery (Murray & Szostak, 1983a). By adding *Tetrahymena* telomeres (TEL elements) to plasmids containing ARS and CEN elements these are able to replicate as linear minichromosomes. These types of plasmids are aptly named yeast artificial chromomsomes (YAC) (Murray & Szostak 1983b; Burke *et al.*, 1987). It has been suggested that yeast artificial chromosomes vectors are more stable than other vectors when transformed into *Physcomitrella patens* (Schaefer 1994). Our experience (**I**) of YAC vectors in *Physcomitrella patens* is that there is no striking effect observed on vector stability when CEN, ARS and TEL elements are included that can be attributed to the presence of these elements.

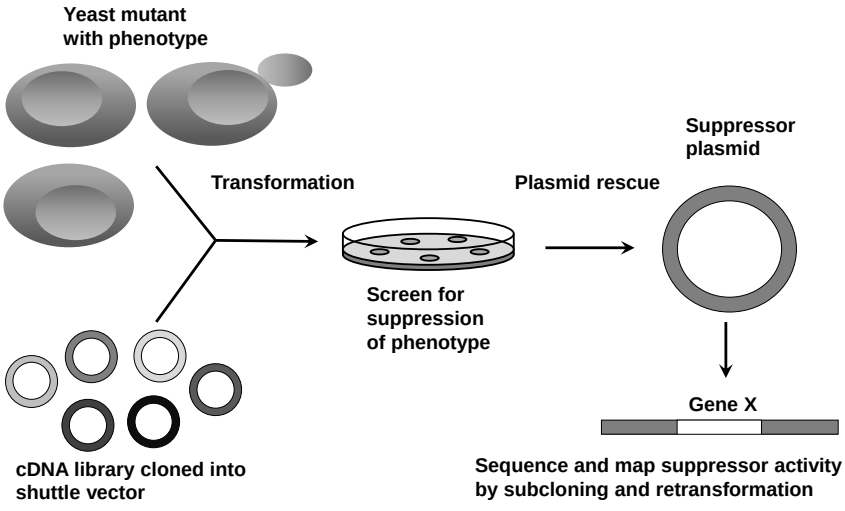

Figure 3. Dosage suppressor screen in yeast,

The importance of yeast shuttle plasmids for eukaryotic molecular genetics cannot be overstated. Shuttle vectors enabled researchers to clone genes by complementation, *i.e.* isolate novel genes by complementing yeast mutants with shuttle vectors carrying a genomic or cDNA library from yeast or from some other organism. Many human genes were first cloned in this way by complementation in yeast. Other important yeast methods using shuttle plasmids include the yeast 2-hybrid and 1-hybrid methods and dosage suppressor genetics, also known as high copy number suppressor screens (Figure 3). In a high copy suppressor screen a yeast genomic library is transformed into a yeast mutant with a known phenotype that is easily scored. The library is usually a 2µ library, so that the genes are overexpressed. Those genes that are able to suppress the phenotype of the mutant will be genes that are somehow functionally related to the mutated or knocked out gene in the yeast mutant. By rescuing the shuttle plasmids from such colonies back into *E. coli* and sequencing the inserts one can find out what genes are functionally related to the gene of interest, *i.e.* the gene that is mutated in the mutant. The yeast dosage suppressor screen is a very powerful tool. Using this method one could in theory unravel an entire metabolic or regulatory pathway in one single screen. Furthermore, dosage suppression frequently mirrors physical interactions between proteins, so it is possible to identify new protein complexes using this method. In our studies (**I**, **IV**) we have shown that plasmids can replicate faithfully in *Physcomitrella patens*. We have also shown that plasmids transformed into *Physcomitrella* can be rescued back into *E. coli*.

This together with the efficient transformation system in *Physcomitrella* suggests the possibility that one could adapt some of the yeast molecular genetic tools discussed above for use in *Physcomitrella*.

3.5 Auxotrophic mutants

Genetic modification or inactivation of genes as well as the controlled expression of genes located on plasmid or through genomic integration lie at the core of modern functional genetics. All of these methods are dependent on the ability to isolate transformed cells through selection and this in turn requires certain marker genes. In yeast there exist a plethora of different selective marker genes, something that enables efficient work with shuttle plasmids. Some marker genes confer resistance to antibiotics or other toxic compounds. Selection in that case works by growing transformants on or in media containing the antibiotic/toxic compound that kills the untransformed cells. This is the standard method used for selection of transformants in plants. For moss, there are at present only four commonly used selective markers and they all work by the principle of conferring resistance to a toxic compound.

There exists an alternative way of selecting for transformants, a method that is the main stay of molecular genetics in *Saccharomyces cerevisiae*. That is the use of marker genes that complement certain nutritional deficiencies. The most common of these are wild type alleles of yeast genes that take part in the biosynthesis of essential amino acids and nucleotides. (Pronk 2002) One example is the *HIS3* gene that encodes imidazoleglycerol-phosphate dehydratase, an essential enzyme catalyzing the sixth step in histidine biosynthesis which converts D-erythro-imidazole-glycerol-phosphate into imidazol acetol-phosphate and H_2O. In yeast, there are several other marker genes coding for genes that catalyze essential steps in amino acid biosynthesis such as *TRP1* for tryptophan biosynthesis, *MET15* for methionine biosynthesis and *URA3* for pyrimidine biosynthesis. Using the marker genes *HIS3*, *TRP1*, *MET15* and *URA3* requires that the plasmids containing markers are transformed into strains were the corresponding gene has been disrupted, and which are thus auxotrophic for the substance in question. These auxotrophic strains need to be grown in the presence of this substance *e.g.* a *HIS3* knockout strain must be grown on media supplemented with histidine unless they are transformed with the *HIS3* marker. Some marker genes can also be counterselected. The *URA3* gene can thus be counterselected with 5-fluoroorotic acid. The product of the *URA3* gene is orotine-5-monophosphate decarboxylase that converts 5-fluoroorotic acid to 5-fluorouracil, a toxic compound that kills the cell.

In plants, some auxotrophic stains have been characterized that are defective in the biosynthesis of essential substrates for instance in *Arabidopsis* where auxotrophs for the amino acids histidine and tryptophan have been studied (Muralla *et al.*, 2007; Last *et al.*, 1991; Last & Fink, 1988). In this thesis it is shown that we could produce auxotrophic lines of *Physcomitrella patens* requring the amino acids histidine and tryptophan, and that we could complement the histidine auxotroph with the native *PpHIS3* gene (**IV**).

4 Energy and carbon metabolism in plants

4.1 Photosynthesis

All life on earth is dependent on the energy that is derived from the sun, and it is plants that provide the basis for the multitude of different organism by being able to capture the energy within sunlight through photosynthesis. In photosynthesis the plant essentially uses the energy of the sunlight to forge carbon-carbon and carbon-hydrogen bonds in organic molecules. The primary carbon metabolism in plants, as in all living organisms, has two main functions, to provide free energy to the plant and to provide a large variety of organic molecules that serve as building blocks for macromolecules. These building blocks are used to produce nucleic acids, proteins and lipids (Knight *et al.*, 2009).

Photosynthesis takes place inside the chloroplasts and can be divided into two parts, the light reaction and the carbon fixation reaction. In the light reaction, light energy is captured and converted into chemical energy by producing ATP from ADP and P_i, in addition to gaining reducing power in the form of NADPH.

The light-capturing reaction occurs in the thylakoid membranes within the chloroplasts, in units called photosystems. The photosystems are divided into an antenna and a reaction center. The chlorophyll in the antenna absorbs the light energy and the chlorophyll *a* molecule uses that energy for the excitation of one of its electrons transferring it to an electron acceptor and thus initiating the electron flow. There are two photosystems, photosystem I and photosystem II, that work together. When the electrons in photosystem II are excited by the light energy as mentioned above the electrons are transferred through the electron transport chain that connects photosystem I and photosystem II.

The chlorophyll *a* molecules in photosystem II can replace the electron by extracting it from water. In this process called photolysis, water molecules are

split, producing protons and oxygen. The protons are released into the lumen of the thylakoids, creating a proton gradient across the membrane that drives the synthesis of ATP by means of an ATP synthase complex located in the thylakoid membrane. The process by which ATP is generated in the chloroplast is very similar to the way ATP is generated by proton driven ATP synthesis in the mitochondria. The oxygen that is produced through photolysis is released into the atmosphere.

The electrons that are transferred through the electron transport chain pass from photosystem I to photosystem II and end up reducing $NADP^+$ to NADPH. The ATP and NADPH produced in the light reaction is then used in the second part of the photosynthesis, the carbon fixation reaction also known as the Calvin cycle. Carbon dioxide (CO_2) enters the Calvin cycle by binding to ribulose 1,5-biphosphate (in C_3 plants), a five carbon sugar with two phosphate groups. Ribulose 1,5-biphosphate is both the starting compound and the end compound of the Calvin cycle, as it is regenerated in each cycle (Raven *et al.*, 1999).

4.2 Production and transport of sucrose in plants

The main product of the Calvin cycle is a triose phosphate called glyceraldehyde-3-phosphate. Glyceraldehyde-3-phosphate is transported from the chloroplast into the cytosol of the cell, or converted through a series of steps into starch and stored in the chloroplast. Exported triose phosphate can be fed into the glycolytic pathway and subsequently into the Krebs cycle where the energy used by the cell in its metabolic reactions is produced. Some of the glyceraldehyde-3-phosphate is also used in different biosynthetic reactions within the cell.

If the production of glyceraldehyde-3-phosphate exceeds the energy demands of the cell the glyceraldehyde-3-phosphate may be converted into sucrose. The sucrose can be stored in the vacuoles or exported to other cells to be used in their metabolism. Sucrose is a non-reducing sugar which makes it an excellent molecule for export to other cells. Much of the carbon that is fixed by the plant is usually converted to starch or sucrose. Starch is usually accumulated in the photosynthetically active tissue during the day time when there is light to drive photosynthesis. At night, the starch is broken down into maltose and glucose which is transported out from the chloroplast, thus providing carbon for continued growth during the night and for cytoplasmic sucrose synthesis (Smith & Stitt, 2007). Starch degradation can be hydrolytic, producing glucose, or phosphorylytic, producing glucose-1-phosphate.

Generally speaking, plant tissue can be divided into two types of tissue, one type is called *Source* tissues and consists of photosynthetically active cells, *e.g.* leaves, that produce an excess of carbohydrates that are then exported to the second type of tissues that are called *Sink* tissues, like roots for instance. Sucrose is the molecule primarily responsible for the energy transport from the source tissue to sink tissues. In higher plants the sucrose is transported through the plant phloem. Depending on the subcellular localization, sucrose is degraded by different enzymes. Sucrose can be degraded by sucrose synthase producing fructose and UDP-glucose, (sucrose synthase also catalyses the reverse reaction, hence its name). Sucrose can also be hydrolyzed by invertases, producing glucose and fructose. There are cell wall invertases, cytosolic invertases and vacuolar invertases, hydrolyzing sucrose at different locations. The ability to produce UDP-glucose directly from sucrose may also be an important reason why plants use sucrose for transporting sugars between cells. UDP-glucose is important because it is used to synthesize cell wall polysaccharides among other things. Another possible advantage of sucrose is that it is not as readily available to potential pant pathogens as *e.g.* glucose (Paul *et al.*, 2008). When sucrose has been broken down into the hexoses glucose and fructose, the latter are phosphorylated into glucose-6-phophate and fructose-6-phosphate so that they can be utilized in different metabolic pathways. This phosphorylation is carried out by the enzyme hexokinase. The phosphorylated hexoses are used in glycolysis which leads into the Krebs cycle. Hexose phosphates are also used in the biosynthesis of nucleic acids and amino acids via the pentose phosphate pathway, additionally hexoses are used in cell wall synthesis.

4.3 Plant growth inhibiting regulatory systems

4.3.1 . SNF1-related Protein Kinase1 (SnRK1)

In plants the SNF1-related Protein Kinase 1 (SnRK1) is an important regulator of energy and carbon metabolism and does the same job as its homologs Snf1 (Sucrose non Fermenting 1) in yeast and AMP-activated kinase (AMPK) in animals. In plants, SnRK proteins can be divided into three subfamilies called the SnRK1, SnRK2 and SnRK3 kinases. SnRK1 are the true orthologs of the above mentioned Snf1 and AMPK kinases whereas the SnRK2 and SnRK3 subfamilies appear to be plant specific.

SNF1, AMPK and SnRK1 are heterotrimeric protein complexes consisting of the catalytic α-subunit and two subunits, the β- and γ-subunits, with regulatory functions. In plants there are also more divergent plant specific γ-

subunits and the AKINβγ subunits (Abe *et al.,* 1995; Bouly *et al.,* 1999; Lumbreras *et al.,* 2001; Slocombe *et al.,* 2002).

SnRK1 acts as regulators that monitors and responds to intracellular energy starvation. In animals, this takes the form of a high AMP to ATP ratio that will activate AMPK whereas in yeast the low glucose levels indicating low energy starvation will activate Snf1 (Rutter *et al.* 2003). In yeast, the Snf1 kinase is activated by ADP instead of AMP, which also signals a low energy status (Mayer et al., 2011). The activation of SnRK1 seems to be dependent on the phosphorylation of a threonine residue on the catalytic subunit. The AMP to ATP ratio does seem to have an effect on the phosphorylation of this residue, high ratio of ATP to AMP tends to inhibit phosphorylation SnKR1 (Sugden *et al.*, 1999a; 1999b) The regulation of SnRK1 is probably a complex process involving many different proteins and metabolites.

Once activated, the Snf1-related kinases will direct the cell to restore its energy level by shutting down anabolic pathways and activating catabolic pathways, it does so in part by direct phosphorylation of enzymes involved in primary cellular metabolism which usually leads to their inactivation. However, SnRK1 also regulates the transcription on genes related to carbon and energy metabolism. Regulation of SnRK1 activity in plants is believed to be independent of the hexokinase signaling pathway discussed below (Rolland *et al.*, 2006; Halford & Hey 2009; Smeekens *et al.*, 2010).

4.3.2 bZIP transcription factors

Transcription factors regulate most biological processes. One family of transcription factor is the basic region/leucine zipper (bZIP) transcription factors. As the name suggests the bZIP transcription factor contains a basic DNA binding region and a leucine zipper dimerization motif. In *Arabidopsis*, bZIP transcription factors regulate many different biological systems from pathogen response to stress signaling and seed maturation. In particular, they have also been implicated in the response to sugar signaling. When it comes to growth regulation it seems that the S1 and C class bZIP transcription factors are of particular interests. The S1 bZIP and C bZIP transcription factors form different combinations of heterodimers that serve as transcriptional activators with a large regulatory potential. (Weltmeier *et al.*, 2006; Weltmeier *et al.*, 2009; Alonso *et al.*, 2007; Hanson *et al.*, 2009). The different bZIP transcription factors are expressed at different levels in response to different metabolic signals. The set of heterodimerized bZIP transcription factors that are expressed will also vary depending on external factors and the cell type. In *Arabidopsis* it is the S1-class bZIP1, bZIP2, bZIP11, bZIP44 and bZIP53, and the C-class bZIP9, bZIP10, bZIP25 and bZIP63 that are part of the growth

regulatory system. Interestingly, all these S1 bZIP transcription factors are also translationally repressed by sucrose (Weltmeier *et al.*, 2009).

Two plant orthologs to the yeast Snf1 kinase called KIN10 and KIN11 have been shown to play an important role in energy metabolism. KIN10 and KIN11 react to energy limitation which causes them to become activated. This in turn activates certain bZIP heterodimers, most likely by direct phosphorylation. The active bZIP heterodimers bind to target genes involved in things like energy consumption, amino acid metabolism and gluconeogenesis, causing activation of transcription and a resulting altered cellular metabolism. Just as the S1 bZIP transcription factors are repressed by sucrose, the KIN10 and KIN11 kinases are also inactivated by high levels of sucrose (Baena-Gonzalez *et al.*, 2007; Hanson *et al.*, 2008; Hanson *et al.*, 2011; Weltmeier *et al.*, 2009). Aside from regulating amino acid metabolism, the above mentioned bZIP11 transcription factors also seems to inhibit plant growth. Interestingly, bZIP11 has been shown to control the trehalose pathway by regulating several of the genes involved in trehalose metabolism, thereby altering the levels of trehalose and trehalose 6-phosphate (Hanson *et al.*, 2011). The picture that emerges suggests a complex cross-talk among the major growth controlling pathways in plants.

4.4 Plant growth promoting regulatory pathways

4.4.1 The target of rapamycin (TOR) kinase pathway

In the 1970s, the bacterium *Streptomyces hygroscopicus* was isolated from a soil sample taken on Easter Island. This bacterium produced an antifungal compound that the researchers named Rapamycin after the local name for Easter Island, Rapa Nui. Experiments in yeast revealed that rapamycin binds to the Frp1 protein and inhibits the TOR kinase (Heitman *et al.*, 1992). All eukaryotic genomes examined have one or more TOR kinases. The TOR signaling pathway is highly conserved and links the availability of nutrients to cell growth. TOR is involved in regulating translation, ribosome biogenesis and the primary metabolism. When there are favorable growth conditions for the cell, TOR is active and positively affects activities that promote growth, but when growth is limited by a lack of nutrients the TOR pathway is inhibited. When the TOR pathway is inhibited protein synthesis is downregulated and genes related to stress resistance are upregulated (Smeekens *et al.*, 2010; Menand *et al.*, 2004). In many organisms, TOR interacts with a protein called Raptor to form the TOR-Raptor complex that activates ribosomal S6 kinase which in turn affects translation. (Tzeng *et al.*, 2009) Interestingly, in mammals AMPK phosphorylates and inhibits the TOR activator protein Raptor (Gwinn

et al., 2008) showing a possible way that the TOR and SnRK1 signaling pathways interact.

4.4.2 Trehalose 6-phosphate signaling

Trehalose consists of two glucose units linked to each other in an α, α-1,1 configuration, this produces a very stable non reducing disaccharide, which can withstand a temperature of 100 °C and a wide pH range. Trehalose can also help to preserve lipid bilayer structure in the absence of water, something that is not observed with close analogues. Unlike sucrose, which is present in large amounts in plants, trehalose is only present in trace amounts. The signaling molecule trehalose-6-phosphate is synthesized from UDP-glucose and glucose-6-phosphate by trehalose-6-phosphate synthase (TPS) at the same time producing uridine diphosphate (UDP). Trehalose 6-phosphate phosphatase (TPP) converts trehalose-6-phosphate to trehalose and inorganic phosphate, trehalase can then convert trehalose to glucose. In plants there are typically many TPS and TPP genes but some are not catalytically active. It has been suggested that catalytically inactive TPS proteins may have some kind of regulatory role in the trehalose pathway (Ramon *et al.*, 2009; Paul *et al.*, 2008) Likewise the TPP genes also seem to take part in signaling and metabolic regulation (Li *et al.*, 2008). In yeast, trehalose-6-phosphate controls the influx of glucose into glycolysis and if the synthesis of trehalose is disabled, the ability to grow on glucose is impaired. In plants, the regulatory role of the trehalose pathway is more complex, but the pathway is a central regulator of plant metabolism. Trehalose-6-phosphate levels in plants are difficult to accurately measure, but high trehalose-6-phosphate levels generally correlates with high levels of sugars such as sucrose. The trehalose-6-phosphate level is inversely related to the amount of UDP-glucose and glucose-6-phosphate (Schuluepmann *et al.*, 2003) which possibly indicates that trehalose-6-phosphate regulates the amount of these substrates. The synthesis of trehalose-6-phosphate could function as an indicator of UDP-glucose and glucose-6-phosphate pool size which in turn reflects the availability of sucrose since sucrose feeds into this pool, at the same time trehalose is not a major end product in plants and would thus not disturb the metabolism. The trehalose pathway seems to regulate multiple developmental and physiological processes like starch accumulation, embryo development, and flowering (Paul *et al.*, 2008; Smeekens *et al.*, 2010).

4.4.3 Hexokinase dependent glucose signaling

In addition to the SnRK, TOR, and trehalose-6-phosphate signaling pathways, there is also a glucose sensing and signaling pathway in plants. This pathway

relies on hexokinases that are able to regulate gene expression in response to glucose and also seem to be involved in extensive cross-talk with signaling pathways that respond to plant hormones. This hexokinase-mediated signaling is believed to be independent of the enzymatic function of hexokinase. Later in this thesis, hexokinase will be discussed in more detail.

4.5 Sucrose and glucose signaling

4.5.1 Glucose repression in *Saccharomyces cerevisiae*

Sugar sensing and signaling has been quite extensively studied in yeast. Glucose is a preferred energy source when it is available for the yeast cell, and yeast will usually consume glucose through fermentation and thus produce ethanol. It will do so also in the presence of oxygen, even though respiration would be more efficient energy wise, the reason presumably being that the ethanol produced inhibits the growth of competing microorganisms. Once the glucose or other sugars have been depleted in the medium, yeast will undergo a major change in the metabolism called the diauxic shift. This allows the yeast cell to use ethanol as a carbon and energy source and thus to continue to grow in the post-diauxic shift phase. When all the ethanol has been consumed, the yeast will cease to grow and enter stationary phase. The change in metabolism during the diauxic shift involves the transcriptional derepression of genes involved in the metabolism of carbon sources other than glucose, genes that are repressed by the presence of glucose in the media. Hexokinase 2 (Hxk2) which is the major hexokinase in yeast in addition to its enzymatic function also plays a role in glucose sensing and signaling. In the absence of glucose, a Hxk2-dependent signal causes a protein kinase named Snf1 to be dephosphorylated and inactivated. When Snf1 is phosphorylated and thus active it mediates derepression of the glucose repressed genes by preventing the transcription factor Mig1 from repressing its target genes. In short, Snf1 phosphorylates Mig1 which causes it to be exported from the nucleus, thus derepressing the glucose repressed genes (Ronne, 1995; Johnston, 1999; Rolland *et al.*, 2002).

In addition to glucose repression of metabolic genes, the concentration of glucose also regulates which type of hexose transporter that are expressed. This makes sense since different hexose transporters have different affinities for glucose. This regulation depends on Snf3 and Rgt2, two transmembrane transporter homologues in the plasma membrane, which act as high and low affinity glucose sensors (Özcan *et al*,. 1996; 1998).

4.5.2 Sucrose signaling in plants

Sucrose has been proposed to be a signaling molecule in plants, but its possible role in signaling is difficult to study since sucrose is easily converted to glucose and fructose, monosaccharides that by themselves trigger signaling. Discerning which molecule is responsible for the effects observed may thus be difficult to elucidate, except in those cases were the sucrose-dependent effects are not seen when plants are treated with glucose or fructose (Rolland *et al.* 2006). The Sucrose transporters SUT2 and SUC3 have been proposed to function as sucrose sensors in plants, similar to the role of the yeast Snf3 and Rgt2 glucose transporter homologues in glucose sensing. However, the role of SUT2 and SUC3 as sucrose sensors is still disputed (Eckardt, 2003).

4.5.3 Glucose signaling in plants

While the need for sugar sensing and signaling is more obvious in a single cell organism like *Saccharomyces cerevisiae* that often has to adapt to a rapidly changes environment, it is no less important for multi-cellular organism, which need to maintain energy homeostasis within their tissues and cells. This maintenance of energy homeostasis is a process that demands constant monitoring and needs to be able to adapt rapidly to changes in sugar concentrations inside the organism.

In multi-cellular organism such as plants, growth and development is a more complex process than in yeast, nonetheless the availability of sugars affects plant growth. A multi-cellular plant needs to coordinate signaling between many cells over long distances. Growth and development is controlled by a complex interplay between the availability of nutrients, plant hormones and other environmental factors. Many plants as previously mentioned contain both source tissues and sink tissues. Generally speaking, photosynthesis, nutrient mobilization and sugar export are upregulated in source tissue when the sugar levels are low. When the sugar levels are high, growth and storage of carbohydrates are upregulated in sink tissues (Rolland *et al.*, 2006).

Most research regarding signaling in plants has been focused on plant hormones such as auxin, cytokinin, gibberellins, abscisic acid and ethylene. There is extensive and complex cross-talk between plant hormones and several plant hormones are often involved in regulating the same processes. Sugar signaling is a subject that is less well understood but has received more interest lately. In screens made with the purpose of looking for sugar sensitive or insensitive mutants in *Arabidopsis* seedlings, several mutants were identified that were allelic to mutants found in abscisic acid and stress response screens. This indicated that sugar is a signaling molecule and that there is some sort of

crosstalk between plant hormones and sugar signaling (Leon & Sheen, 2003). Interestingly, it further seems that the intracellular concentrations of sugars can affect the response. Increased cell wall invertase activity in the *Arabidopsis* meristem thus leads to accelerated flowering and an increase in seed yield. If there on the other hand is increased expression of cytoplasmic invertase, this leads to deflowering and a lower seed yield (Heyer *et al.*, 2004).

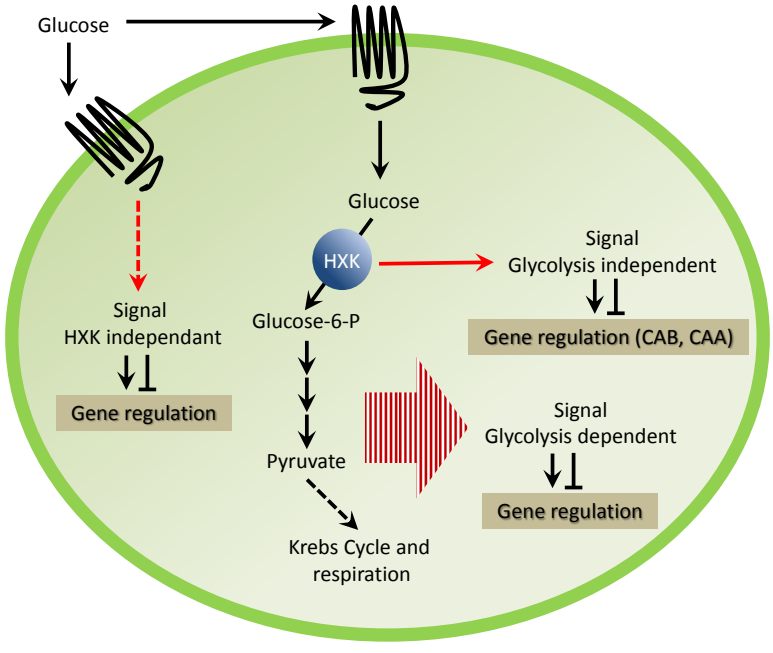

Figure 4. In plants there are three proposed ways by which glucose signaling may occur.

Three are at least three types of glucose signaling observed in plants (Figure 4). The first is the type that has already briefly been touched upon and that is the hexokinase mediated signaling that is independent of the enzymatic activity of hexokinase (Moore *et al*., 2003). This pathway regulates photosynthetic genes that are repressed by glucose such as ribulose-1,5-bisphosphate carboxylase small subunit (*RBCS*), the chlorophyll a/b-binding protein (*CAB*) and carbonic anhydrase 2 (*CAA*). There is also a glucose signaling pathway that is reliant upon the enzymatic function of hexokinase. What is actually being sensed is either the glucose-6-phosphate directly produced or a downstream product formed in glycolysis, or perhaps even the ATP (Xiao *et al.*, 2000). This pathway is illustrated by the PR1 and PR5 genes responding to yeast HXK2 expressed in *Arabidopsis*. Thirdly, we have glucose signaling pathways that are completely independent of hexokinase, as illustrated by the glucose dependent

regulation of genes such as phenylalanine ammonia-lyase (PAL1), aspargine synthase (ASN1), chalcone synthase (CHS), and AGPase, which does not respond to yeast *HXK2* being overexpressed in *Arabidopsis* nor to expression of either sense or antisense *AtHXK1* constructs (Xiao *et al.*, 2000).

One glucose insensitive *Arabidopsis* mutant that has been extensively studied is the glucose insensitive 2 mutant (*gin2*) (Moore *et al.*, 2003), When this mutant was characterized it was found to be mutated in hexokinase 1 (AtHXK1). The conclusion that could be drawn once the *gin2* mutant had been studied were that the phenotypes could be explained by defective glucose signaling. AtHXK1 thus appears to play a key role in sugar sensing and signaling in *Arabidopsis*. In 2006, it was further shown that small amounts of AtHXK1 translocate to the nucleus (Cho *et al.*, 2006a) giving indications as to how AtHXK1 may exert its effect on transcriptional regulation.

5 Hexokinases

Hexokinase is an enzyme that phosphorylates hexoses, more specifically glucose into glucose-6-phosphate and fructose into fructose-6-phosphate. There are also kinases that are specific for either glucose (yeast glucokinase) or fructose (plant fructokinases). The latter are not related to the hexokinases, but yeast glucokinase is a member of the hexokinase family.

The hexokinase enzyme is usually made up of two identical subunits. When glucose or fructose binds to the hexokinase, the two subunits undergo conformational changes that allow the binding of ATP and subsequent phosphorylation of the substrate (Kuser *et al.*, 2000).

Hexokinase catalyzes the first step in glycolysis by phosphorylating glucose into glucose-6-phosphate. Glycolysis is a sequence of enzymatic reactions that turns glucose into pyruvate with the simultaneous production of a small amount of ATP and NADH providing the cell with energy. As mentioned earlier, the pyruvate produced by glycolysis is fed into the Krebs cycle during aerobic growth. Glycolysis also produces several three and six carbon intermediate compounds that are useful for other cellular processes.

5.1 Subcellular localization of hexokinases

In plants as in other organisms, many proteins exists in different isoforms, this is also true for the hexokinases. Different plant hexokinases are targeted to different intracellular compartments, depending on their N-terminal sequences (Olsson *et al.*, 2003). In particular, type A hexokinases possess a chloroplast transit peptide and localize to the chloroplast stroma, whereas type B hexokinases possess an N-terminal membrane anchor and localize to mitochondrial and chloroplast membranes (Olsson *et al.*, 2003).

In *Arabidopsis*, there are six members of the hexokinase family. They have been divided into two groups, one group are the “true” hexokinases AtHXK1,

AtHXK2 and AtHXK3. The second group is the hexokinase-like proteins AtHKL1, AtHKL2 and AtHKL3 that do not seem to be able to phosphorylate hexoses (Karve *et al.*, 2008). All the HXKs and HKLs have an N-terminal transmembrane anchor except HXK3, the single type A hexokinase *Arabidopsis*, that instead has a chloroplast transit peptide and localizes to the chloroplast stroma. There are ten hexokinases in rice (*Oryza sativa*), OsHXK1 through OsHXK10. OsHXK4 localizes to the chloroplast stroma, OsHXK7 localizes to the cytosol and the rest of the hexokinases in rice are membrane bound hexokinases (Cho *et al.*, 2006b). So it is clear that different hexokinases are associated with different compartments in the cell (Figure 5). This is consistent with subcellular fractioning and biochemical studies where hexokinase activity has been shown to be associated with different compartments such as mitochondria, plastids and cytosol (Claeyssen & Rivoal, 2007).

In rice, almost all of the hexokinase genes are expressed in most tissues such as the leaves, the flowers, the roots and immature seeds. An exception is OsHXK10 which appears to be pollen specific (Cho *et al.*, 2006b). Given that there are so many hexokinases in rice it would be interesting to know if most of these hexokinases are redundant or have unique functions in the cell or in the plant.

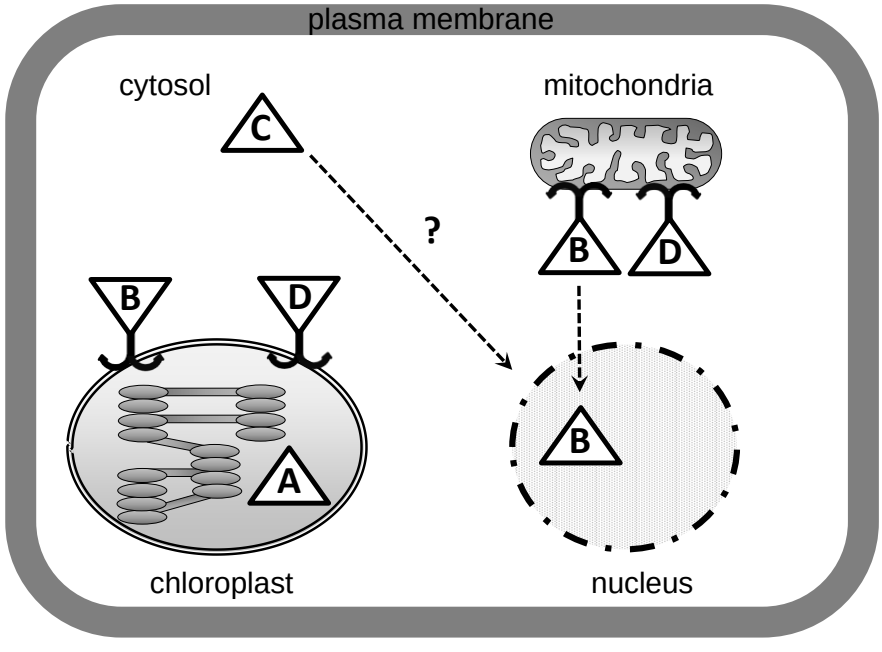

Figure 5. Subcellular localization of hexokinases in plants.

In tobacco, there are nine hexokinases, NtHXK2 is the single type A hexokinase (Karve *et al.*, 2010). NtHXK1, NtHXK1a, NtHXK3, NtHXK4a, NtHXK4b, NtHXK5 and NtHXK7 are type B hexokinases that associate with the mithochondria. There also exists a hexokinase, NtHXL1, that lacks glucose phosphorylating activity (Kim *et al.*, 2013).

In *Physcomitrella*, eleven hexokinases have been annotated. The localization of the different hexokinases is determined by their N-terminal sequences (**II**). The *Physcomitrella* hexokinases can be divided into four different groups based on these sequences. The three type A hexokinases localize to the chloroplast stroma (PpHxk1, PpHxk5 and PpHxk6). The four type B hexokinases possess a transmembrane region (PpHxk2, PpHxk3, PpHxk7 and PpHxk8) that targets them to mitochondrial and chloroplast membranes. The single type C hexokinase (PpHxk4) lacks an N-terminal signal peptide and localizes to the cytosol. Lastly, the three type D hexokinases (PpHxk9, PpHxk10 and PpHxk11) also possess transmembrane anchors but these are different in sequence from those in the type B hexokinases. PpHXK1 is the *Physcomitrella patens* hexokinase that has been most extensively studied. It was the first hexokinase to be found to localize to the chloroplast stroma. It is also the major hexokinase in *Physcomitrella* accounting for 80% of the glucokinase and 47% of the fructokinase activity in protonemal tissue (Olsson *et al.*, 2003; Thelander *et al.*, 2005). Type A and B hexokinases are also found in vascular plants (see above), whereas the type C and D hexokinases so far have been found only in *Physcomitrella*.

It should be noted that in *Arabidopsis*, the membrane bound hexokinases seem to localize to the mitochondria exclusively (Karve *et al.*, 2008). In contrast, the spinach (*Spinacia oleracea*) hexokinase SoHXK1 was reported to localize to both chloroplast and mitochondrial membranes (Wiese *et al.*, 1999), however a later publication claimed that this enzyme localizes only to the mitochondria (Damari-Weissler *et al.*, 2007). In *Physcomitrella*, type B and D hexokinases seem to associate with both mitochondrial and chloroplast membranes (**I**).

5.2 Hexokinase as a glucose sensor

As already mentioned, there is increasing evidence that hexokinases serve a key function in a glucose sensing and/or signaling pathway in plants. This started with the discovery that overexpression of AtHXK1 and AtHXK2 in *Arabidopsis* causes hypersensitivity to glucose, whereas plants that expressed antisense constructs were hyposensitive to glucose (Jang *et al.*, 1997).

Additional evidence was added when AtHXK1 turned up in a screen for glucose insensitive mutants in *Arabidopsis*, the so called *gin2* mutant. To test if the *gin2* phenotype was the result of the hexokinases enzymatic activity or not, an experiment was performed that sought to test whether or not the mutant phenotype could be rescued using a hexokinase that lacked catalytic activity. The result from this study showed that a transgenic *gin2* mutants transformed with a catalytically inactive hexokinase restored the wild type glucose response. The conclusion was that the *gin2* phenotype was not due to a lack of the enzymatic activity of AtHXK1, instead it was postulated that AtHXK1 is part of signaling pathway that is independent of glucose phosphorylation (Moore *et al.*, 2003).

Later it was shown that small amounts of AtHXK1, estimated to roughly 1 % of cellular AtHXK1 is localized to the nucleus. Through the use of a yeast two hybrid screen coupled with proteomics, two proteins were shown to interact with the AtHXK1 in the nucleus. These two proteins were RPT5B, a subunit of the 19s regulatory particle in the proteasome, and the protein VHA-B1 which is the vacuolar H^+-ATPase B1. Neither of these proteins had previously been implicated in any sort of glucose signaling (Cho *et al.*, 2006a). It would thus appear that AtHXK1 localizes to the nucleus as part of a multiprotein complex. It is rather surprising that AtHXK1 which is a type B hexokinases and hence membrane bound is able to translocate to the nucleus. The mechanism by which this translocation occurs is so far not understood. It has been suggested that there could be some kind of membrane exchange or even direct membrane contact between different organelles inside the cell (Claeyssen & Rivoal, 2007). There is also some evidence that the glucose signaling via hexokinase is connected to F-actin. Large alterations of cellular F-actin can be observed in response to glucose treatment, which is one of the earliest noticeable responses. Furthermore, it has been shown that for AtHXK1 to act as a glucose sensor, an actin cytoskeleton that is normal and functioning is needed. In addition to this it has also been shown that AtHXK1 may interact with F-actin (Balasubramanian *et al.*, 2007; Balasubramanian *et al.*, 2008). It should be noted that Balasubramanian *et al.*, (2007) used the same antibody as Cho *et al.* (2006) but were unable to detect AtHXK1 in the nucleus, possibly due to the fact that they started with a much smaller amount of plant tissue.

There is also evidence from other plants that substantiates the hypothesis that hexokinase mediates glucose signaling. Thus, the NtHXK1 and NtHXK1a proteins in tobacco, which appears to be two alleles of the same gene, may play the same role as AtHXK1 in glucose signaling as evidenced by their ability to complement the gin2-1 mutant (Kim *et al.*, 2013). In rice, at least two hexokinases are thought to be involved in sugar sensing and signaling:

OsHXK5 and OsHXK6. A study was performed were OsHXK5 and OsHXK6 were fused to green fluorescent protein (GFP) and transiently expressed in maize protoplasts. OsHXK5 and OsHXK6 localized to the mitochondria membrane as one would expect. However, when using GFP constructs where the N-terminal mitochondrial targeting peptide was removed, the proteins localized primarily to the nucleus, with only small amounts being present in the cytosol. Importantly, OsHXK5 and OsHXK6 as well as their catalytically inactive mutant alleles were shown to complement the *Arabidopsis gin2* mutant, restoring a wild type phenotype to seedlings. Finally, overexpressing OsHXK5 and OsHXK6 in rice caused hypersensitivity to glucose, leading to growth retardation and repression of the photosynthetic gene RbcS (Cho *et al.*, 2009).

5.2.1 D-allose induced growth inhibition dependent on hexokinase

Arabidopsis hexokinase 1 has, aside from its catalytic ability and its role in glucose sensing, also recently been implicated in D-allose induced growth inhibition. D-allose is rare epimer of D-glucose. D-allose seems to affect transcription by suppressing gibberillin responsive genes and inducing expression of ABA-related genes including the bZIP transcription factor OSABF1. D-allose inhibits growth, but this growth inhibition was not observed in the *gin2-1* mutant. Furthermore, *gin2-1* expressing wild type AtHXK1 recovered its sensitivity to D-allose in contrast to *gin2-1* expressing AtHXK1 enzymatically inactivated due to a point mutation in its phosphoryl transfer site. This suggests that the D-allose growth inhibition unlike glucose signaling is dependent on phosphorylation of D-allose via hexokinase (Fukumoto *et al.*, 2013).

5.3 Hexokinase and programmed cell death

Hexokinases also play a role in programmed cell death (PCD). PCD, also known as apoptosis, is distinct from necrosis which results from tissue damage and from autophagy which is lysosomal degradation of proteins and organelles. As the name implies, PCD is a process that is very tightly regulated. It was already known from mammals that release of cytochrome c from the mitochondria into the cytoplasm of the cell is one of the first steps in PCD, this was also shown to be true for plants (Balk *et al.*, 1999). The release of cytochrome c is dependent on a pore complex, the permeability transition pore, located in the mitochondrial membrane. This pore complex transports ions and different metabolites out of the mitochondria. Hexokinase was shown to interact with the voltage-dependent anion channel (VDAC), a protein within

the pore complex. Binding of hexokinase to VDAC closes the pore and prevents release of cytochrome c, resulting in inhibition of apoptosis (Azoulay-Zohar *et al.*, 2004). In a study using tobacco (*Nicotiana benthamiana*) it was shown that a mitochondria-bound hexokinase (NtHxk1) plays a similar role as previously seen for hexokinases in mammalian cells. Virus induced gene silencing of NtHxk1 in tobacco thus causes PCD. In contrast, overexpression of mitochondrial hexokinase (AtHXK1 and AtHXK2) in *Arabidopsis* blocks PCD under normally PCD-inducing conditions (Kim *et al.*, 2006).

6 Aims

This thesis had two different aims, the first of which was to acquire a deeper understanding of carbon metabolism in plants, and more specifically the role of plant hexokinases, their characteristics and localization, as well as their possible role in glucose signaling. The second aim was to broaden the tools available for the plant model system *Physcomitrella patens* by trying to adapt tools from yeast molecular genetic to *Physcomitrella*.

6.1 Paper I

That *Physcomitrella* possesses a native system for homologous recombination that allows for precise gene targeting is well established (Cove, 2005). It is also known that transformed DNA can replicate episomally in *Physcomitrella* (Schaefer, 1994; Ashton et al., 2000). Developing a shuttle plasmid system for *Physcomitrella patens* that is similar to those used in yeast would be a great advantage for molecular genetics in plants. With this in mind the aim of this study was to test three different plasmids transformed into *Physcomitrella* in order to determine their stability. Specifically, we wanted to check if certain features on a plasmid like yeast autonomously replicating sequences (*ARS* elements) or yeast centromeres (*CEN*) and telomeres (*TEL*) could help to stabilize the plasmid. Finally, and most importantly, we wanted to characterize episomally replicating DNA in more detail, and investigate if this DNA can be rescued back into *E. coli*.

6.2 Paper II

PpHxk1 was the first hexokinase discovered and characterized in *Physcomitrella* (Olsson *et al.*, 2003; Thelander *et al.*, 2005). Since there is no evidence that PpHxk1 is involved in glucose signaling, one objective of this

study was to characterize the other hexokinases in *Physcomitrella* and to figure out which hexokinases were most likely to be involved in glucose signaling. We also wanted to get a better understanding of the evolution of the plant hexokinase family. This work was made substantially easier by the sequencing of the *Physcomitrella* genome. Given that *Physcomitrella* as well as other plants have multiple hexokinases it seemed obvious that some of the hexokinases could have different functions within the cell. We believed that the function of a plant hexokinase in part would depend on its subcellular localization. To observe their localization *in vivo* we decided to use transient expression analyses with GFP (green fluorescent protein) fused to *Physcomitrella* hexokinase sequences. Our hope was that this localization study would provide clues to which hexokinases are potential candidates to being involved in glucose sensing and signaling and/or PCD.

6.3 Paper III

Work with the glucose insensitive mutant *gin2-1* suggested that *Arabidopsis* hexokinase 1 (AtHXK1) functions as a glucose sensor. Moreover it appeared that this glucose signaling is independent of the enzymatic function of the hexokinase, since a catalytically inactive hexokinase still retained its signaling function (Moore *et al.* 2003). Later, evidence was also presented that rice has the same kind of hexokinase-dependent signaling as present in *Arabidopsis* (Cho *et al.* 2009), thus suggesting that hexokinase mediated glucose signaling may be present in a wide range of plants. Our aim was to see if the charophyte microalgae *Klebsormidium nitens* also might possess hexokinase mediated signaling as seen in land plants. The objective was to characterize the *Klebsormidium nitens* hexokinase 1 (KnHxk1) and to express it in both yeast and *Arabidopsis* hexokinase mutants in order to test its ability to complement both hexose phosphorylation and glucose signaling defects.

6.4 Paper IV

The use of auxotrophic strains in the model system *Saccharomyces cerevisiae* has greatly facilitated yeast molecular genetics work. Multiply auxotrophic strains are usually used as genetic backgrounds for work with plasmids containing marker genes. The marker genes are wild type alleles that complement the defect of the mutant auxotrophic yeast strain (Pronk, 2002). One example of this type of marker gene is the *HIS3* gene that encodes imidazoleglycerol-phosphate dehydratase. *HIS3* is an indispensible enzyme catalyzing the sixth step in histidine biosynthesis. We were interested in seeing

if this type molecular genetic tool could be adapted for use in *Physcomitella patens*. The aim of the study was to knock out the *HIS3* homologue in the *Physcomitrella* genome and then test if we could complement the mutant strain with the wild type allele expressed from a plasmid. We also wanted to show that this type of plasmid could be rescued from the moss back into *E. coli*.

7 Results and discussion

7.1 Paper I

To investigate the stability and fate of plasmids transformed into *Physcomitrella* we constructed three different plasmids, pEM203, pEM207 and pEM209. All plasmids contained an *E.coli* origin, Amp^R gene and the *nptII* gene used for selection of moss transformants. In addition, pEM207 contained yeast *ARS* and *CEN* elements, and pEM209 contained yeast *ARS*, *CEN* and *TEL* elements. These elements were derived from the yeast artificial chromosome vector pYAC4. Both the original circular and linearized versions of the plasmids were transformed into *Physcomitrella*. Initially, we found that the circular plasmid gave more transformants but after 8 weeks most of these circular transformants had lost the capability to grow on selection plates, and the transformants obtained with linearized DNA were more numerous. We could not observe any effects on the stability of episomal replication that could be attributed to the *ARS* and *CEN* elements. We could, however, see that pEM209, which contained *TEL* elements, produced more stable transformants than pEM203 and pEM207. This is most likely due to increase in plasmid integration into chromosomal DNA and not due to increased stability of episomal replication. It is possible that the repetitive DNA within the *TEL* element more readily integrates into the genome.

Importantly, we could show that plasmids transformed into *Physcomitrella* can be rescued back into *E. coli* from undigested *Physcomitrella* DNA. Furthermore, plasmids rescued from moss transformed with circular plasmids were identical to the original plasmid. Linearization of the plasmid DNA before transformation increased the number of stably replicating transformants. Plasmids rescued from these moss transformants were either identical to the original plasmid, or carried deletions in which the site used to linearize the DNA had been lost. The deletions seem to have arisen due to direct repeat

recombination in the moss. The original plasmids, and sometimes several plasmids with different deletions, were frequently recovered from the same moss transformants. It is likely that such mixed populations arise because the transformed moss takes up several copies of the linearized plasmid which are then repaired in different ways.

Through Southern blot we could see that episomal replication of linearized DNA was associated with a more than 1000-fold amplification. Transformants obtained with circular plasmids had much lower copy numbers. Stable chromosomal integrants containing a resistance marker never lose their resistance. In contrast, the episomal transformants quickly lose the plasmid with the resistance marker if selection is removed. However, we also recovered a third type of stable episomal transformant that resembles chromosomal integrants but still loses the plasmid at a low but detectable frequency. The reason why some episomal transformants are highly stable in moss is unknown. It has been suggested that transformed DNA could pick up pieces of moss DNA and that these pieces might promote plasmid replication and/or stability, in the same way that the *ARS* and *CEN* elements do in yeast (Schaefer, 1994). However, we did not observe any presence of moss DNA in the highly stable episomal transformant in our study. We speculate that a possible explanation for the highly stable episomal transformants is instead rare integrations into accessory mini-chromosomes. Such mini-chromosomes are known to be present in mosses (Newton, 1984). If these mini-chromosomes are non-essential and just fortuitously replicating selfish DNA, they would be subject to occasional loss by missegregation.

7.2 Paper II

The novel chloroplast localized type A hexokinase in *Physcomitrella*, PpHxk1, had already been characterized (Olsson *et al.*, 2003; Thelander *et al.*, 2005). To follow up this work we set out to characterize all members of the hexokinase protein family in *Physcomitrella*. By PCR-cloning, several new hexokinase transcripts were found, additional transcripts were also found in publicly available databases for ESTs. The latter transcripts were either PCR amplified from cDNA or ordered from the Japanese RIKEN collection. Full length transcripts were found for most of the hexokinases, but for some hexokinases we could only find partial or aberrantly spliced transcripts. In total, we found ten different hexokinases in *Physcomitrella*. Later, when the first genome sequence of *Physcomitrella patens* was made publicly available, eleven putative hexokinase genes were identified. Judging from the intron-exon organization and the conserved splice sites we believe that all eleven

hexokinase genes may encode functional proteins. As mentioned above, there is proof that at least ten of these hexokinase genes are expressed. The fact that we have not found any transcripts from one of the genes, *PpHXK6*, could be due to it being expressed only under specific conditions or developmental stages. The *PpHXK11* encoded protein has a few important amino acid substitutions that possibly could affect its catalytic activity. Curiously, we found that aberrantly spliced hexokinase cDNAs were common. We also found an interesting case of alternative splicing for *PpHXK7*, where a splice variant had lost the sequences encoding the membrane anchor. We speculate that this alternative splicing could make it possible for the PpHxk7 protein to enter the nucleus and there affect transcription.

The *Physcomitrella* hexokinases can be grouped into four different subfamilies. The three type A hexokinases (PpHxk1, PpHxk5 and PpHxk6) contain a chloroplast transit peptide, the four type B hexokinases (PpHxk2, PpHxk3, PpHxk7 and PpHxk8) possess an N-terminal membrane anchor, the single type C hexokinase (PpHxk4) has neither a transit peptide nor a membrane region, and finally the three type D hexokinases (PpHxk9, PpxkK10 and PpHxk11) also possess N-terminal membrane anchors but are different in sequence from the type B hexokinases; the type D hexokinases also differ in their intron-exon organization from the type B hexokinases. Expression analyses of hexokinases fused to GFP showed that all three type A hexokinases as expected localize to the interior of the chloroplasts. The type B and type D hexokinases, on the other hand, localize to the chloroplast and mitochondrial membranes. Interestingly, some hexokinases have a dual localization to both the chloroplast and the mitochondrial membrane. The single type C hexokinase in *Physcomitrella* localizes primarily to the cytosol. The fact that there are so many different hexokinase isoforms could be due to different hexokinases having different roles in the same cell or to the different hexokinases having different developmental or tissue specific expression. More work need to be done in the future to elucidate the roll of the different *Physcomitrella* hexokinases.

7.3 Paper III

Klebsormidium nitens is a small single celled microalga that belongs to the charophytes, the closest relatives among the algae to the land plants. It possesses a single hexokinase that we named KnHxk1. From the sequence of KnHxk1 it is clear that it is a type B hexokinase with a N-terminal membrane anchor. By fusing the open reading frame of KnHxk1 to sequences encoding the GFP protein and expressing this construct transiently from a plasmid in

Physcomitrella protoplasts, we could show that KnHxk1 localizes to the mitochondria, but if the membrane anchor is removed, it remains in the cytosol. To investigate if KnHxk1 is capable of transmitting a glucose signal as seen in higher plants we made transgenic lines based on the *Arabidopsis* glucose insensitive mutant *gin2-1* background (Moore *et al.*, 2003). These transgenic lines contained the *KnHXK1* gene expressed from the 35S promotor. Three types of transgenic lines each expressing different variants of the *KnHXK1* gene were made. The first variant was the unaltered *KnHXK1* wild type allele, the second variant was the *KnHXK1* gene but lacking the sequences encoding the membrane anchor. Finally, the third variant was the full *KnHXK1* gene but altered by two point mutations, the first mutation in the ATP binding region and the second mutation in the phosphoryl transfer site. Both point mutations are by themselves believed to render the hexokinase catalytically inactive. To see if the *Klebsormidium nitens* hexokinases could complement the *gin2-1* mutant we grew the transgenic seedlings on 6% glucose, which causes growth inhibition in wild type plants. The results showed that the wild type KnHxk1 could restore the wild type phenotype to the *gin2-1* mutant. Similarly, KnHxk1 lacking the N-terminal membrane anchor could also restore the wild type phenotype. To our surprise, the catalytically inactive mutant version of KnHxk1 could not restore the wild type phenotype. This is in contrast to previous results indicating that the glucose signaling mediated by *Arabidopsis* hexokinase 1 is independent of its enzymatic activity (Moore *et al.*, 2003). The same pattern of complementation and non-complementation of the transgenic lines could be observed in high light and low light experiments. The result of RT-PCR on glucose repressed genes in *Arabidopsis* confirmed these observations. One possible important difference between catalytically inactive KnHxk1 and previously tested catalytically inactive hexokinases (Moore *et al.*, 2003; Cho *et al.*, 2009) is that our mutant carried both point mutations in the same gene whereas in previous experiment, two mutants were tested each carrying only one point mutation. It is possible that both mutations in the same protein may disrupt the glucose signaling function by destabilizing the hexokinase protein or the signaling complex. An alternative interpretation would be that the catalytic function in previous experiments was not fully eliminated by only one point mutation, and that glucose signaling is actually dependent on the enzymatic activity of hexokinase. Finally, we also investigated whether wild type and mutant versions of KnHxk1 could complement the hexose phosphorylation and glucose signaling defects in a yeast *hxk1 hxk2 glk1* triple-mutant strain lacking all hexokinase activity. These experiments proved that KnHxk1 is an active hexokinase able to phosphorylate

both glucose and fructose, and that it could complement the glucose signaling defect also in yeast.

7.4 Paper IV

Auxotrophic *Physcomitrella patens* strains were generated by fully removing the single gene, *PpHIS3*, encoding imidazoleglycerol-phosphate dehydratase, which catalyzes the sixth step in histidine biosynthesis from the moss genome, creating a *PpHIS3*Δ knockout strain. Similarly, the *PpTRP1* gene encoding phosphoribosylanthranilate isomerase, an enzyme that catalyzes the third step in tryptophan biosynthesis, was removed from the moss genome, creating a *PpTRP1*Δ knockout strain. The knockout strains were tested for their ability to grow on media lacking histidine and tryptophan, respectively. The results showed that the *PpTRP1*Δ strain was unable to grow without externally supplied tryptophan, and similarly the *PpHIS3*Δ strain was unable to grow without histidine added to the growth media. In contrast, when the *PpHIS3*Δ strain was grown on media containing 250 µM of histidine the *PpHIS3*Δ strain grew and behaved as the wild type strain.

With the goal of adapting yeast molecular genetic tools to *Physcomitrella* we preceded to test if the *PpHIS3*Δ strain could be complemented using a plasmid with the wild type *PpHIS3* gene expressed from the 35S promotor, This plasmid was transformed into moss in both intact circular and linearized form. Both forms of the plasmid could complement the *PpHIS3*Δ strain. The fate of the constructs was investigated by PCR analysis. In most cases, the linearized plasmid integrated as concatemeres into the moss genome via recombination between the 35S promotor present in the knockout construct and the 35S promoter in the knockout selection marker previously integrated into the moss genome. In contrast, the circular plasmid seemed not to integrate into the genomic copy of the 35S promotor. Finally, we found that the complementing plasmid could be rescued from moss back into *E. coli* indicating that cloning by complementation and other methods that rely on the use of shuttle vectors may be feasible in *Physcomitrella*.

8 Conclusions and Future Perspectives

8.1 Hexokinases and glucose signaling in *Physcomitrella patens*

It is likely that one or more of the eleven hexokinases in *Physcomitrella* is involved in glucose signaling as observed in *Arabidopsis* with AtHxk1. The problem is figuring out which hexokinase is fulfilling the glucose sensing role. The glucose signaling described in *Arabidopsis* involves a membrane bound hexokinase. If this is the case also in *Physcomitrella*, possible candidates would be PpHxk2, PpHxk3, PpHxk7, PpHxk8, PpHxk9, Ppxk10 and PpHxk11, all of which have N-terminal membrane anchors. Since it is a type B hexokinase that is involved in glucose signaling in *Arabidopsis*, the safest bet would be to investigate the type B hexokinases in *Physcomitrella*. In this context, the alternative splicing of PpHxk7 that produces a variant lacking the N-terminal membrane anchor makes it a prime candidate for further investigations. Generating knockout of the type B hexokinase genes starting with *PpHXK7* and testing for glucose signaling would be a good start in further investigating the hexokinases in moss and their roll in glucose sensing and signaling. One can speculate that this naturally occurring splice variant could be part of a control mechanism involved in glucose signaling. It would be interesting to see if the glucose concentration in the environment of the cell affects this splicing and possible localization of *PpHXK7*.

Data gained from studying knockouts of *PpHXK3*, *PpHXK4*, *PpHXK5* and *PpHXK6* (data not included in this thesis) shows no indication that *PpHXK3*, *PpHXK4* or the type A hexokinases are functioning as glucose sensors. Furthermore, determining if all of the hexokinases are functional hexokinases could be done ether via complementation of the yeast triple *hxk1, hxk2, glk1* or via enzymatic assay. In Arabidopsis there are several catalytically inactive hexokinase like genes.

8.2 Identifying novel hexokinase interacting proteins

A second interesting study would be to look for new proteins interacting with the glucose sensing hexokinase(s). The glucose signaling function of the hexokinase AtHXK1 in *Arabidopsis* is dependent on its interaction with several other proteins, which together form a nuclear complex (Cho *et al.*, 2006a). It is therefore reasonable to believe that any hexokinase in *Physcomitrella* that directs glucose signaling by translocating to the nucleus and influencing transcription does so as part of a complex. If this were to be the case it would point to a conserved mechanism for glucose sensing in plants. The use of a yeast two-hybrid screen to search for hexokinase interacting proteins in *Physcomitrella* could shed new light on how moss hexokinase affects transcription and which partners it interacts with. However, any such studies would first require that moss hexokinases involved in glucose signaling are identified (see above).

8.3 Hexose phosphorylation and glucose sensing

The biggest question raised by the study of the *Klebsormidium nitens* is why the catalytically inactive form of KnHxk1 did not restore glucose sensing to the *gin2-1* mutant. The first thing to test would be to see if this is specific to the *Klebsormidium nitens* hexokinase 1 or not. The same type of double point mutation that we introduced in KnHxk1, affecting both the ATP binding region and the phosphoryl transfer site, should be introduced in the AtHxk1 protein of *Arabidopsis*. This mutated AtHxk1 should then be transformed into the *gin2-1* mutant line to see if the AtHxk1 double mutant is still able to transmit the glucose signal, both on a phenotypic level and on the transcriptional level.

8.4 Developing a shuttle vector for *Physcomitrella*

One of the plasmids that was obtained in the first study of plasmids in *Physcomitrella* in Paper II is most likely what Schaefer (1994) termed a class III transformant, which is thought to represent episomal transformants that are highly stable. We have concluded that this stability is most likely not due to some additional DNA being picked up from the moss genome that promotes plasmid replication and/or stability. Instead, we speculate that the highly stable episomal transformants represent integrations into one of the non-essential mini-chromosomes that are known to be present in moss (Newton, 1984). The plasmid could be integrated as a concatemere into the mini-chromosome. To verify that our class III transformant is integrated we will cleave the moss DNA with a restriction enzyme that cuts the plasmid once. When the restriction

enzyme cleaves the DNA it will therefore release several copies of the plasmid as well as a few plasmids that contain the flanking regions of the locus where the concatemere was integrated. The cleaved moss DNA will be circularized by ligation and then transformed into *E. coli*. Most of the plasmids rescued in this way should thus contain only plasmid DNA but some should carry the flanking sequences. Any flanking sequences found will be sequenced and compared to the *Physcomitrella* genome. We will then proceed to make a knockout construct that targets a resistance marker to this locus to see if we obtain the same type of highly stable transformant that resembles chromosomal integrants but still loses the plasmid at a low but detectable frequency. This would be an indication that class III transformants are indeed integrated into mini-chromosomes that are subject to occasional loss by missegregation. Further studies of moss mini-chromosomes might also facilitate the development of YAC type vectors that have greater stability and replicate more efficiently in moss.

8.5 Cloning by complementation and dosage suppressor screens

One very tantalizing prospect that would increase the value of*Physcomitrella* as a plant model system, is if cloning by complementation could be performed in the moss. The last paper in this thesis shows that a mutant *Physcomitrella* strain can be complemented by the wild type allele expressed from a plasmid. The next step would be to transform a mutant strain with a moss cDNA library and see if it is possible to find transformants containing the correct complementing wild type gene. The PpHIS3Δ knockout strain is good candidate for such a proof of principle experiment since it is already known that it can be complemented, and is easy to score due to being an auxotroph. If cloning by complementation can be shown to work, the next step would be to do a dosage suppressor screen. One possible screen that could be carried out would use a *snf1a snf1b* double knockout mutant that is hypersensitive to auxin and unable to grow in low light conditions (Thelander *et al.*, 2004) making the mutant easy to score. The *snf1a snf1b* double knockout would be transformed with a *Physcomitrella* cDNA library. The transformed moss would then be grown under conditions where the double mutant fails to grow. Any surviving moss colonies should contain either one of the two knocked out genes or a suppressor gene, *i.e.* another gene that suppresses the mutant phenotype when overexpressed. Plasmids will be rescued from the surviving colonies and the inserts identified by sequencing. These experiments would not only prove that

suppressor screens in *Physcomitrella* are possible, but we might also gain useful information about the functions of PpSnf1 and its regulatory role.

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Acknowledgements

And here I am at last writing the final part in my thesis, the point when it's time to turn on the sentimental tap and let it rip. Over the years I have met many amazing people and experienced so many things. How different my life is now at the end of this journey compared to the way it was at the beginning. I would firstly like to thank all the people that who have helped me during my time as a PhD student and who have contributed to the work presented in this thesis and to the work not presented in this thesis.

I would especially like to thank **Hans Ronne** my supervisor. Sitting listening to you talking about yeast and moss as a guest lecturer in the course Genome function, I would never have guessed that I would be sitting here years later writing my thesis. I thank you for giving me the opportunity to do scientific research and for being an inspiring supervisor, researcher and scholar. My life is better for having known you and that statement goes beyond purely academic pursuits.

I would also like to thank my co-supervisor **Mattias Thelander** for always having times for questions and for all the great input and expertise. For starting the *Physcomitrella* legacy in the Ronne lab that I in the end inherited. To the rest of the moss people I had the pleasure of working with, **Eva M.** for being dependable and solid, you are a shining example of how the craft of laboratory work is supposed to be practiced. **Monika J.** your smiles and good mood always brightened up the lab. **Zhen,** who came into the moss lab only at the end when all others had left, your help was truly invaluable, I don't think I could have made the final push without your help.
I would especially like to thank **Anders,** for showing me the ropes, for all your help along the way which would make a list that is far too long to recount and for simply being an all-around great guy.

Mattias C. for each and every time making all our theories and speculations about science and far beyond a blast. **Niklas N.** thank you for your help in the lab and your great sense of humor, for helping me keep my sanity, together with **Gustav**, **Adam**, **Johan** and **Mats** you helped me remember what's truly important ;-) *Alea iacta est!*

Other people in the D9:3 lab for help and humor **Karolina**, **Frida**, **Gunilla** och **Jenny**.

Nils Elfving for visiting our lab and proving that mutants can have phenotypes. **Emma Hörnblad**, working with you was a joy. You raised my spirit at a time when things felt pretty bleak. **Magnus Eklund** thank you for your help and support allowing me to step outside my laboratory comfort zone. **Gunilla Swärdh** for all your help with *Arabidopsis* and always taking time to answer my pesky questions.

A big thank you to all the great **colleges** at the Microbiology department, **Ingmar**, **Anki**, **Stina** and **Sture** for practical and administrative support. Special thanks to the people in the EM group **Volkmar**, **Ievgeniia, Johanna** and **Tomas,** not seeing you every Tuesday and in the lab is going to be weird.
I would also like to thank the people at IMBIM/Uppsala University in particular the people I met in **IPhAB**, also **Rashmi** and **Freyja** for making my time spent teaching students so memorable.

Manou I finally completed this thing and delivered it as promised. By the way, your plant is still alive :-)

Christine det kommer alltid finnas en plats vid vårt köksbord för dig.

Johan A., **Orla**, **Johan K**. and **Chris**, we don't see each other as often as I'd like, sometimes it seems like London might as well be the surface of the moon. To both Johan's that IKEA table was the best god damn table I ever had the pleasure of building, thank you for all the laughs through the years.

Maria och Bosse tack för er omtanke och stöd, inte minst på slutet då Sanna och jag verkligen behövde det.
Erika och **familj** tack för er gästfrihet, ni har alltid fått oss att känna oss så välkomna i Sjöfallet.

Lars, **Nina** och **barnen** tack för att ni är så fina vänner. Lars min barndoms kompis, en trogen vän och en god människa tack för alla lyckliga minnen.

Tack **Mamma** och **Pappa,** jag kunde inte ha önskat mig ett bättre par föräldrar än er. Tack för allt ert tålamod, möda och kärlek som jag inte kunnat uppskatta fullt ut innan jag fick egna barn.
Stort tack till **Johan, Anna** och **Wilgot**. Min kära storebror Johan du har alltid funnits där när jag har behövt det.

Till **Egil**, **Ada** och **Sigrid** för att ni invaderade och erövrade mig och hela min värld och gjorde mig för evigt tacksam över detta.
Till **Susanna** tack för ditt stöd, din förståelse och din kärlek. Att förklara vad du och barnen betyder för mig ligger bortom alla ord, om jag inte hade dig vore inget möjligt.