SUGAR SENSING

IN

SPIRODELA PUNCTATA

by

Cynthia M. Boettger

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Plant and Soil Sciences

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To Cate my shining star!

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Abstract

The angiosperm *Spirodela punctata* (*Lemnaceae*) can utilize glucose and adapt to galactose as the sole carbon source for heterotrophic growth. Two partial cDNAs were isolated from *S.punctata* by RT-PCR using degenerate primers. SpGAL1 is a 312bp fragment that encodes galactokinase (E.C. 2.7.1.6), and SpHXK a 612bp fragment that encodes hexokinase (E.C. 2.7.1.1). QPCR expression studies showed that SpHXK mRNA transcriptional gene expression levels are always up-regulated regardless of the carbon source or the time of sampling. We were unable to test for changes in SpGAL mRNA expression levels.

Chapter 1

Introduction

Saccharomyces cerevisiae prefers glucose and other closely related sugars as a carbon source of choice because it converts rapidly to ethanol (Rolland, 2001). When *S. cerevisiae* uses glucose as a carbon source the genes involved in fermentation and growth are up-regulated, while the genes for metabolism of alternative carbon sources such as galactose are down-regulated; this regulatory mechanism is known as catabolite repression (Johnson, 1999, Carlson, 1999, Ullman, 1996). Alternatively, when galactose is used as a sole carbon source, galactose can affect the down-regulation of enzymes previously repressed by glucose (Gancedo, 1998).

In plants, the products of photosynthesis are mainly sucrose, fructose and glucose (Sheen, 1999, Smeekens, 1999). Unlike *S. cerevisiae* which can utilize galactose as an alternative carbon source, free galactose is extremely toxic to higher plants.

This toxicity is due to the fact that galactose-1-phosphate (EC 5.3.1.26) inhibits the enzyme phosphoglucomutase and consumes ATP (Physiology Hexoses and Pentoses).

It has been observed that the angiosperm *Spirodela punctata* (*Lemnaceae*) can utilize either glucose or galactose as a sole carbon source for heterotrophic growth (Frick, 1994). This aquatic plant can assimilate each of these hexoses under continuous light and dark growth conditions (Frick, 1994).

Hexokinase and galactokinase are the first enzymes of the glucose and galactose utilization pathways. In order for *S. punctata* to utilize either glucose or galactose to drive hetrotrophic growth in continuous darkness it would seem plausible that the plant uses hexokinase and galactokinase as sugar sensors. In order to test this idea we would like to use *S. punctata* for a classic catabolite repression study, but must first demonstrate that this angiosperm

employs catabolite repression in regulating carbohydrate metabolism.

We feel that *S. punctata* is the proper choice as a model system for this study for several reasons: *S. punctata* is a monocotyledonous aquatic angiosperm from the duckweed family. *S. punctata* reproduces vegetatively by sequential budding from intercalary meristems to produce daughter fronds. Our studies feature *S. punctata* for a number of reasons: 1) the free-floating plants are small, < 5mm long by < 4mm wide and can be manipulated easily in Erlenmeyer flasks, 2) *Spirodela* can be grown vegetatively, aseptically, heterotrophically and perpetually under relatively constant conditions of temperature with weekly brief light pulses, and heterotrophy is supported by a variety of reduced carbon sources (Frick, 91), 3) The plant cultures grow log-linearly, shift readily and reversibly between both carbon sources and between phototrophy (illuminated, no reduced carbon source) and heterotrophy (continuous darkness with assimilatable carbon sources) (Frick,

1976), 4) All vegetative phases of development are represented in a small quantity of tissue (Frick, 1976).

It has been reported that ***Lemna minor*** callus uses both the Leloir and Isselbacher pathways to assimilate galactose, and that galactokinase specific activity is up-regulated in the presence of galactose (Frick, 1995).

Due to the fact that ***Spirodela*** can utilize either glucose or galactose as the sole carbon source in continuous darkness we are postulating that ***S. punctata*** is using the Leloir and Isselbacher pathways in much the same way that ***Lemna*** does (Frick, 95).

Our study is interested in hexose metabolism that is responsive at the transcriptional level only. We postulate the following: When ***S. punctata*** is growing on glucose as a sole carbon source we have a classic model of catabolite repression (transcription level inhibition of a variety of inducible enzymes by glucose), as seen in ***Saccharomyces cerevisiae*** (Gancedo, 1998). In the simplest of terms when glucose is the

sole carbon source for *S. punctata* growing in continuous darkness, hexokinase (HXK) mRNA should be up-regulated and galactokinase (GAL1) mRNA should be down-regulated.

The opposite should also hold true: When *S. punctata* is growing on galactose as a sole carbon source in continuous darkness we should see GAL1 mRNA up-regulated and HXK mRNA down-regulated.

For our transcriptional study we use QPCR (quantitative polymerase chain reaction) in an attempt to show that: 1) *S. punctata* does employ a classic catabolite repression model since it can utilize both glucose and galactose as sole carbon sources for growth in continuous darkness. 2) If our results show that *S. punctata* is displaying a classic catabolite repression model then hexokinase can not be the only sugar sensor of plants.

Chapter 2

Literature Review

2.1 GAL gene introduction

The galactose utilization pathway (Fig.1) has been extensively studied in the budding yeast, *Saccharomyces cerevisiae* (Ideker, 2001). This yeast has provided a model system to study eukaryotic gene regulation in reference to galactose utilization as a sole carbon source.

The Gal genes are a set of regulatory and structural genes that are rigidly regulated at the transcriptional level via the carbon source. When *S. cerevisiae* is given D-galactose as the sole carbon source the GAL genes are activated and encode the enzymes of the Leloir pathway (Barnett, 2004).

When the Gal genes are activated they allow the yeast to use galactose for growth and metabolism

(Timson, 2002, Melcher and Xu 2001). In the presence of glucose the Gal genes are inert (Platt, 2000). The Gal structural genes include GAL2 which encodes galactose permease and the 3 Leloir enzymes GAL1, galactokinase, GAL7, galactose-1-phosphate-uridylytransferase and GAL10, UDP-galactose-4-epimerase, which convert UDP-galactose into UDP-glucose. These enzymes have been conserved throughout evolution from bacteria to mammals (Christacos, 2000).

The regulatory genes encode 3 proteins Gal4p the transcriptional activator, Gal80p the repressor, and Gal3p the inducer. These proteins determine the transcriptional status of the genes needed for galactose utilization (Bhat and Murthy, 2001).

2.2 The Gal Structural Genes

2.2.1 Gal2p, Galactose permease

In *S. cerevisiae* GAL2 encodes the galactose transporter protein Gal2p. Gal2p transports galactose

into the cell and belongs to a family of short-lived plasma membrane proteins (Horaket, 2001). Gal2p is tightly regulated, so that maximal activity is achieved when cells are growing on galactose as a sole carbon source. When either glucose or any other easily fermentable sugar is added to the growth medium GAL2 transcription is repressed and the Gal2p transporter is inactivated by a process referred to as catabolite repression (Matern and Holzer, 1977). This glucose related process is thought to speed up the cells transition from galactose to the preferred carbon source glucose (Horak, 2001).

2.2.2 Gal1p Galactokinase

Galactokinase (E C 2.7.1.6) the product of the GAL1 gene has 2 functions in the galactose pathway. First, Gal1p interacts with Gal80p (repressor protein) by activating Gal4p (transcriptional activator of the GAL regulon). The function of Gal1p can also be performed by Gal3p which is also a transcriptional

activator (Vollenbroich, 1999). Although Gal1p can perform this function it does so at a 40 fold less efficient rate than Gal3p (Platt, 2000). Second, galactokinase catalyzes the formation of galactose-1-phosphate (Vollenbroich, 1999).

Galactokinase was first isolated from mammalian liver (Holden, 2003) and has been studied in bacteria (Veerhees, 2002) yeast (Schell and Wilson, 1977) humans (Reece and Timson, 2003) and plants (Dey, 1983).

Galactokinase deficiencies in humans lead to galactosemia II which is characterized by the early-onset of cataracts (Holden, 2003).

The function of galactokinase in plants is not well understood. In plants the majority of galactose is in the cell walls in the form of (1-4) B-galactan. It is also found in the glycan portion of the cell wall. Glycoproteins are a minor component of hemicelluloses. Galactose is also present in the galactolipids of the chloroplast membrane (Kaplan, 1997).

2.2.3 Gal10, UDP-galactose-4-epimerase

UDP-galactose-4-epimerase (E.C.5.1.3.2) or "galactowaldenase" functions in the interconversion of UDP-glucose and UDP-galactose (Lake, 1998). Through a series of reactions in the Leloir pathway, over-expression of Gal10 gives bacteria and some plants the ability to metabolize galactose as a sole carbon source. UDP-galactose-4-epimerase catalyzes the final step in normal galactose metabolism by regenerating UDP-glucose (Holden, 2003 and Majumdar, 2004).

In *E.coli*, mutants of UDP-galactose-4-epimerase can not catabolize galactose as a sole carbon source. The bacteria accumulate toxic levels of galactose intermediates. This accumulation of toxins would indicate an essential role for UDP-glucose-4 epimerase in galactose catabolism (Lake, 1998).

UDP-glucose-4 epimerase in plants functions primarily to maintain the appropriate levels of UDP-

glucose and UDP-galactose for cell metabolism rather than for galactose utilization (Soffer, 1962).

2.2.4 Gal-1-p-uridyltransferase

Galactose-1-phosphate uridylytransferase (EC 2.7.7.12) catalyzes the interconversion of galactose-1-phosphate and glucose-1-phosphate. This is the second step in the Leloir pathway of galactose metabolism (Christacos, 2000).

2.3 The GAL regulatory genes

2.3.1 Gal4P

The GAL4 gene encodes a protein of 881 amino acids (Johnson, 1992), and is the transcriptional activator of the GAL regulon. When Gal4p binds to the upstream activation sequence (UAS_{gal}) in the nucleus of the cell the GAL genes are turned on (Peng and Hopper, 2002).

In *S. cerevisiae* when galactose is present in the growth medium as the sole carbon source Gal3p liberates the repression of Gal80p over Gal4p. This in turns enables the expression of the structural genes Gal1, 2, 7 and 10 (Wu, 1996).

When glucose is present in the growth medium of *S. cerevisiae* GAL gene transcription is repressed even if galactose is present (Adams, 1972). The repressing effects of glucose could act on Gal4p several different ways. It could prevent synthesis by disabling the ability to bind to the UAS_{gal}, or simply interfering with the activating function of the protein. Glucose also interferes with the release of any inhibitory effects the Gal80p has over Gal4p (Johnson, 1994). It has also been observed that in the presence of glucose Gal80p prevents the phosphorylation of Gal4p (Sadowski, 1996).

When glucose is present, transcription of the GAL gene complex is prevented by the nuclear localized Mig1 protein. The Mig1/Tup1/SSn6 protein complex prevents GAL1 and GAL4 genes from initiating

transcription by binding to specific upstream repression sites (URS) (Ostergaard, 2000).

2.3.2 Gal80p

Gal80p is the repressor protein of the GAL genes. Gal80p is present in both the cytoplasm and the nucleus of the cell (Peng and Hopper, 2002).

When *S. cerevisiae* is growing in the absence of galactose the Gal4p is inhibited by the binding of Gal80p to the C-terminal transcription activation domain of Gal4p at amino acid 768-881 (Wolfe and Shields, 1977). When galactose is present Gal80p binds to Gal3p in the cytoplasm and now Gal4p can activate transcription of the Gal genes (Verma, 2003). This allows *S. cerevisiae* to metabolize galactose as a sole carbon source.

2.3.3 Gal3p

The Gal3 encoded protein is a residue protein when in the presence of galactose activates the GAL genes by relieving the repression of Gal80p. In *S.cerevisiae* Gal3p is a 520 amino acid long protein (Murthy and Bhat, 2000), and resides in the cytoplasm of the cell (Peng and Hopper, 2002).

Gal3p is highly related to the galactokinase of the Leloir pathway Gal1p. Gal3p has a 73% identity at the nucleotide level and 92% similarity at the amino acid level in *S. cerevisiae* (Timson, 2002).

Yeast Galactokinases are bi-functional proteins. The protein possesses both kinase and signal transduction activity (Bhat, 1990 and Meyer, 1991). Based on homology region III of *S. cerevisiae* the sequence of Galactokinase was compared with *Escherichia coli, Bacillus subtillis, Candida albicans, Haemophilus influenzae, Salmonella typhimurium, Kluyveromyces, Arabidopsis thaliana, Homo sapiens and Streptomyces lividans.* These sequence alignments

showed that Gal3p has a truncated version of the galactose signature sequence GLSSSA (A/S) (F/L/I). The Gal3p truncation is found at amino acids 161-166 of the protein. Gal3p can be converted to a functional galactokinase if SA is inserted in the Gal3p sequence. This insertion will now enable Gal3p to be a bi-functional protein like galactokinase Gal1p (Platt, 2000).

It has been suggested that GAL3 evolved from GAl1 in order to provide a non-catalytic regulator. When *S.cerevisiae* is shifted to galactose medium then GAL3 is already present in sufficient quantities to bind to galactose and transmit a signal to Gal80p (Melcher, 1997).

2.4 Glucose and Plants

Glucose is universally utilized by plants as a respiratory carbon source. Glucose provides plants with the energy needed to support cell functions as

well as building materials for cell walls, membranes, organelles and proteins (Sheen (sc), 2003).

Sugar production via photosynthesis and its effects on plant metabolism have long been studied, whereas the effects of sugars on sensing and signaling in plants are still very novel. Hexokinase, (EC 2.7.1.1) the enzyme that catalyzes glucose to glucose-6-phosphate (the first step in glycolysis) appears to be at the forefront of study as a glucose sensor in plants (Sheen, 99; Smeekens, 00). One of the difficulties of studying glucose sensing is trying to separate glucose effects on regulatory function from those of nutrient function (Rolland, 2001).

2.5 Glucose Analogs

The question remains how do we know that hexokinase is a sugar sensor? Hexose sugars such as glucose can affect gene expression upon entry into the plant cell or through metabolism activation through the action of the enzyme hexokinase. The sugar

analogs 3-O-methylglucose and 2-deoxyglucose have given evidence of hexose signaling associated with glucose transport (Smeekens, 1998 and Klein, 1998).

3-O-methylglucose is transported into the cell, but is not phosphorylated by hexokinase. Since 3-O-methyl glucose is not phosphorylated by hexokinase the glucose related genes are not turned on (Cortes, 2003).

When some plants are given the glucose analog 2-deoxyglucose it can be phosphorylated by HXK (hexokinase) but not metabolized by the plant. Expression levels of HXK mRNA appear to change when 2-deoxyglucose is supplied to the plant. This glucose analog can induce expression of genes that are required for glucose utilization, while repressing genes for the use of alternative carbon sources (Graham, 1994 and Jang, 1994).

The use of these sugar analogs appears to be a valid and effective way to indicate the role that hexokinase plays in sugar sensing in plants. The effects of sugar analogs should be interpreted with caution as they are not the only means of studying

hexokinase activity. The sugar analogs 2-deoxyglucose and 3-O-Methylglucose also can be toxic to the plants (Rolland, 2002).

2.6 **SNF-1 and Mig1 effects on Catabolite Repression**

Catabolite repression is a regulatory mechanism that is carried out by virtually all living organisms from plants, animals, yeast and down to the simplest of bacteria (Saier, 1998). When glucose levels are high, transcription levels in ***S. cerevisiae*** are repressed for such functions as respiration and the ability to use alternative carbon sources such as galactose. Some genes are induced when glucose concentrations are low, including low affinity glucose transporters and glycolytic enzymes (Carlson, 1999).

The Sucrose Non-Fermenting catalytic subunit (SNF-1) protein kinases are distributed and conserved throughout yeast, plants and animals. The SNF-1 of yeast and the SNF-1 related kinases of plants (SnRK's) have a high sequence similarity to each other. It has

been shown that SnRK's of plants can completely suppress a mutational defect of yeast SNF-1 (Lovas, 2003).

SNF-1 kinase activates and catalyzes the phosphorylation of Mig1 (Kaniak, 04). "Mig 1 is a zinc finger protein that binds to the promoters of numerous glucose-repressed genes" (Carlson, 1998). The main job of SNF-1 kinase is to inhibit Mig1 repression when glucose is limited in the cell, thus controlling the major pathway for glucose repression in yeast (Chronakis, 2004). Mig1p localization is determined by glucose levels. When glucose levels are high Mig1 is imported into the nucleus. When glucose levels are low, Mig1p is transported back to the cytoplasm (Carlson, 1998). As glucose levels become more limiting the phosphorylation of Mig1 increases and is dependent on Snf1 (Wu, 1998; DeVit, 1997 and Johnston, 1994). Snf1 is present in both cytoplasm and the nucleus, but its mechanism is not known (Carlson, 1999).

2.7 Hexokinase

The ATP-dependent phosphorylation of glucose to form glucose-6-phosphate is the first reaction of glycosis, which is catalyzed by the cytosolic isoenzyme hexokinase. The phosphorylation of hexokinase has two purposes: 1) hexokinase converts nonionic glucose into an anion that is trapped in the cell since cells lack transport systems for phosphorylated sugars. 2) Glucose which is inert becomes activated into an unstable form capable of further metabolism.

2.7.1 Plant Hexokinase

Plant hexokinase (HXK) is proposed to be an enzyme with catalytic and regulatory function that has been hypothesized to be involved in sugar sensing and signaling (Jang 1994 and Xiao, 2000).

To show the role that hexokinase plays in sugar sensing two *Arabidopsis* hexokinase cDNA's have been cloned, AtHXK1 and AtHXK2. In transgenic plants over expressing AtHXK we see permanent stunting of development when grown on 6% glucose under constant light. Conversely when AtHXK is under expressed we see normal development when plants are grown on 6% glucose under constant light. Both scenarios hold true for AtHXK1 and AtHXK2 which have a high nucleotide homology to each other of 82% (Jang, 1997; Jang and Sheen, 1997).

It has also been proposed that the rate of hexokinase metabolism may directly correlate with the production of ATP. If this hypothesis holds true for glucose metabolism in plants the hexokinase would be a rate-limiting enzyme and not a "sugar sensor" (Halford, 1999).

2.7.2 Yeast Hexokinase

In the yeast *Saccharomyces cerevisiae* HXK2 is the protein that has a vital role in glucose repression and triggers intracellular glucose metabolism. HXK2 acts as a transcriptional repressor. It has been shown that 15% of the protein is located in the nucleus (Randez-Gil, 1998 and Herrero, 1998). HXK2 is a transcriptional repressor that is found in the nucleus of the cell. During glucose dependent repression nuclear HXK2 is involved in the formation of specific DNA-protein complexes (Herrero, 1998 and Rodriguez, 2001). In high glucose conditions Mig1 and HXK2 are present in the nucleus repressing their promoter regions. When glucose levels are low Mig1 and HXK2 are both phosphorylated and exported out of the nucleus. In the absence of the Mig1/HXK2 complex in the nucleus transcription of glucose regulated genes are up-regulated (Ahuatz, 2004).

Although the role of HXK2 as a putative glucose sensor has been identified, it still remains to be

shown what actually triggers the glucose signal for glucose repression (Rolland, 2002). "Since the rate of glucose transport and phosphorylation correlate well with the level of glucose repression, glucose-6-p or other initial glycolytic metabolites have often been proposed to be the triggering molecules" (Jiang and Carlson, 1996 and Vincent, 1998).

2.8 Quantitative Polymerase Chain Reaction (QPCR)

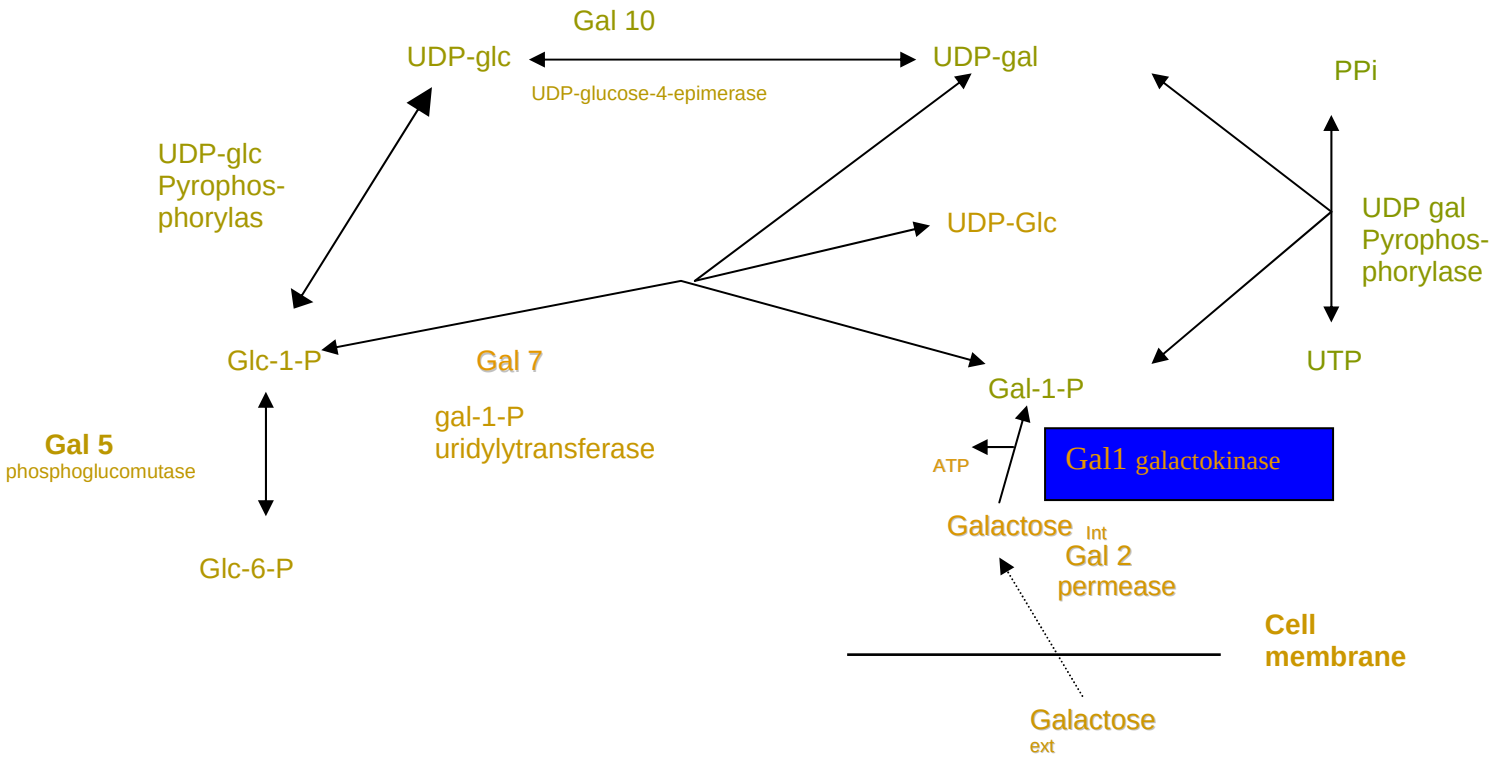
QPCR is the method of choice for quantification of mRNA expression that may be expressed at very low levels. The QPCR is far superior to that of Northern blotting due to its sensitivity, specificity, reliability and speed of gathering data. Another advantage to QPCR is that it requires very small amounts of starting material, which is beneficial when sample sizes are, limited (Gachon, 2004).

Like traditional PCR, QPCR uses forward and reverse primers to amplify a specific amplicon using Taq Polymerase. In addition to the traditional

components, QPCR uses a fluorescent probe to measure the amount of product formed after each round of replication reaction. These fluorescent probes can be non-specific and fluoresce when associated with double stranded DNA. Probes can also be sequence specific. Sequence specific probes can be more costly because they only measure the amount of specific product of interest and will not associate with any extraneous products such as primer dimers. Specific and non-specific probes will emit fluorescence when excited by a laser after each cycle. The choice of chemistry depends on the machine available for use and well as the specificity needed for the assay. This project used SYBR® due to its economical advantages and availability of the ABI 7900 detection system. The SYBR® molecule is an intercalating agent that has high affinity for double stranded DNA (Gachon, 2004, Giulietti, 2001 and Ginzinger, 2002).

Figure 1. Extracellular glucose is transported into the cell via Gal2. Several enzymatic steps convert galactose into glucose-6-phosphate which can then be used by the cell. Gal2, galactose permease; Gal1, galactokinase; Gal7, galactose-1-phosphate uridylytransferase; Gal 10, UDP-glucose-4-empirase; Gal 5, phosphoglucomutase. UDP gal pyrophosphorylase is the enzyme of the Leloir pathway that converts Ga1-P to UDP-gal (Frick,Personal Comm).

Leloir and Isselbacher Pathways



Chapter 3

Materials and Methods

3.1 RNA Extractions for Cloning:

In order to observe galactokinase and hexokinase mRNA expression in *S. punctata* plants, *S. punctata* were grown aseptically in Frick medium (mineral salts and organic supplements) (Frick, 1991) with either 2% galactose or 2% glucose respectively as the sole carbon source in continuous darkness. Once per week the plant cultures were irradiated (600 µmol m^{-2} s^{-1} PAR mixed cool white fluorescent and incandescent light) for 3 minutes (Frick and Morley, 95). Plants were washed briefly in sterile water, frozen in liquid nitrogen and stored at -80 ° C. Total RNA was isolated using an adaptation of a one-step guanidine isothiocyanate separation (Chomczyuski and Sacchi, 1989). Two grams of frozen plants were ground into a

fine powder in liquid nitrogen using a mortar and pestle. The powder was suspended in 10ml of 4M guanidine isothiocyanate, 25mM sodium citrate, 0.5% sarkosyl, 0.1 M β-mercaptoethanol. One ml of 2M sodium acetate, 10ml of buffered saturated phenol and 2ml of 24:1 chloroform: isoamyl alcohol was added to the mix. After cooling on ice, and centrifuging for 20 minutes at 95000 rpm, the aqueous layer was removed to a fresh tube and precipitated with isopropanol. The isopropanol precipitation was repeated and the RNA pellet washed with 70% ethanol, dried and resuspended in 1.0 ml sterile water. OD 260/280 was measured to determine nucleic acid concentration and purity. mRNA was purified from total RNA using Amersham-Pharmacia Biotech Kit (Piscataway, NJ; Kit # 27-9258). Protocols for mRNA processing were described by the manufacturer (Amersham-Pharmacia Biotech).

3.2 Cloning of a Galactokinase and Hexokinase gene fragment from *S. punctata*

We wanted to amplify homologous cDNA fragments from *S. punctata*. It was felt that having our QPCR analysis primers developed from homologous cDNA clones, the perfect primer/mRNA base sequence homologies involving short nucleotide stretches might prove crucial to mRNA detection.

3.2.1 Galactokinase Gene

The *Arabidopsis thaliana* GAL1 mRNA (accession #AF024623) and soybean (*Glycine Max*) galactokinase mRNA accession (#AW507994) were aligned using The CLUSTAL W (3) package of DNAStar: DNAStar, Inc; MegAlign; Version 1.03, 1993 Madison WI., and the conserved areas were used to design degenerate oligonucleotides to prime for RT-PCR reactions (Figures 2A and 2B). The sequence areas chosen correspond to the conserved amino acid region of 545-

563 for (accession #AF024623) and 184-202 for (accession#AW507994) for the forward primer. The reverse primer areas chosen were 749-771 for (accession # AF024623) and 301-323 for (accession #AW507994). The primers flanked the coding region for the conserved galactokinase signature motif of **GLSSSAA** (Platt, 2000) (Fig. 2A and 2B, pg.54). The primers were obtained from Operon (Valencia, CA).

Forward GALK1 5′ CTTGTTGATGGAAYWGTGCCKACAGGTTC 3′)

Reverse GALK2 (5′ GTRACMGCCTTYTGMGACTCTGC 3′).

3.2.2 Hexokinase Gene

The degenerate PCR-primers used were designed with reference to the hexokinase amino acid sequences of (Menu, 2001) (Fig. 3A and 3B, pg.54) for tomato (*Lycopersicon esculentum*) Hxk2 (accession # LeHxk-AF208543), potato (*Solanum tuberosum*) Hxk1 (accession # StHXK1-X94302), *Arabidopsis thaliana* HXK1 (accession # AtHXK-U28214) and *Arabidopsis thaliana* HXK2

(accession # ATHXK2-U28215) hexokinase related amino acid sequences were aligned (Menu et al., 2001) and the conserved areas were used to design degenerate oligonucleotides to prime for HXK using RT-PCR. The nucleotide sequence chosen corresponded to the conserved amino acid region of 62-68 for the forward primer and 280-286 for the reverse primer (Figures 3A and 3B). These primers were obtained from Sigma Genosys (St.Louis, Missouri).

Forward HXK, (5′ ATGCANGTNGARATGCAYGC 3′)

Reverse HXK, (5′ TTNCCCCAYTCCATRTTDATNAC 3′).

3.2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions using these primers were done using a Qiagen one-step RT-PCR kit (# 210210). The 50ul reaction consisted of 10ul 5x

reaction buffer, 2ul Qiagen enzyme mix, 2ul dNTPs (10mM mix), 1 ul of each primer (50 pmol each), 2ul Qiagen enzyme mix, 2ul mRNA 32 ul water. Galactokinase thermocycler parameters were 50° C 30 min, 95° C 10 min, followed by 30 cycles of 94° C 1 min, 60° C 1 min, 72° C 1.5 min, then 72° C for 10 min. Hexokinase thermocycler parameters 50°C 30 min, 95° C 15 min, followed by 30 cycles of 95° C 1 min, 50° C 30 sec, 72 °C 1.5 min then 72° C 10 min. RT-PCR amplified fragments were separated on 1.5% agarose gels. One PCR fragment per reaction was observed. The galactokinase reaction produced a 314 bp band and the hexokinase reaction revealed a 610 bp band.

3.2.4 Cloning and Analysis

Bands were cut from the agarose gel and the fragments were purified using Qiagen Qiaquick gel extraction kit (# 28704) per manufactures instructions.

The purified cDNA fragment was then cloned into pCR2.1 vector using Invitrogen TA cloning Kit (#2000-01). Colonies were selected and processed for sequencing using Qiagen Mini-prep spin kit (#27106). Dye-terminator cycle sequencing was performed on each of the inserts using the same gene-appropriate primers used for RT-PCR. The reactions were run in a single lane on an ABI model 371 automated DNA sequencer. Chromatographic data were analyzed using seqed. 1.03 software ABI.

3.3 Quantitative Polymerase Chain Reaction (QPCR)

Four time-course experiments were performed involving shifts between glucose and galactose as carbon sources, and QPCR to establish SpGAL1 mRNA and SpHXK mRNA expression levels.

3.3.1 Short Carbohydrate Shift, Time-Course One

Time points and growth conditions for time course one. Plant material used for this time course had been grown aseptically in continuous darkness for 3 weeks in Frick medium with a final concentration of either 2% glucose (Sigma G7528) or 2% galactose (Sigma G6404). The galactose used in this experiment contained a minimum of 98% galactose.

Zero-time-points were taken from plants growing in 2% glucose or 2% galactose to establish a baseline signal for SpGAL1 mRNA and SpHXK mRNA transcriptional expression levels.

Plants were also switched from growing on either 2% glucose or 2% galactose to the opposite carbon source. This was done to see how long it would take for the plant to start using the opposite carbon source through either the down-regulation of SpGAL1 mRNA (plants shifted to glucose) or SpHXK mRNA (plants shifted to galactose) to an up-regulation of SpGAL1 mRNA (plants growing on galactose) or SpHXK

mRNA(plants shifted to glucose). Plants were sampled at 4, 8 and 24 hours. All plants were washed briefly in sterile water and frozen in liquid nitrogen and stored in the -80° freezer.

3.3.2 Short Carbohydrate Shift, Time-Course Two

Time points and growth conditions for time course one. Plant material used for this time course had been grown aseptically in continuous darkness for 3 weeks in Frick medium with a final concentration of either 2% glucose (Sigma G7528) or 2% galactose (Sigma G6404). The galactose used in this experiment contained a minimum of 98% galactose.

Plants growing in 2% galactose or 2% glucose were switched to medium containing a final concentration of 2% galactose plus 2% glucose. This was done to see if *S. punctata*, given a choice of carbon sources, would prefer one. Plants were sampled at 4,8,24 hours. All

plants were washed briefly in sterile water and frozen in liquid nitrogen and stored in the -80° freezer.

3.3.3 Long Carbohydrate Shift, Time-Course Three

Time points and growth conditions for the time course three. Plant material used for this time course had been grown aseptically in continuous darkness for 3 weeks in either Frick medium with a final concentration of 2% glucose (Sigma G7528) or 2% galactose (Sigma G0750). The galactose used in this experiment contained a minimum of 99% galactose and contained < 0.01% glucose.

Zero time-points were taken from plants growing in 2% glucose and 2% galactose. This was in an effort to establish a baseline signal for SpHXK mRNA and SpGAL1 mRNA, using either glucose or galactose as the sole carbon source in continuous darkness.

Plants were also switched from growing on 2% glucose to 2% galactose. This was done to see how

long it would take for the plant to start using the opposite carbon source through up-regulation of SpGAL1 mRNA (plants growing on galactose) to a down-regulation SpHXK mRNA (plants shifted to galactose). Plants were sampled at 48, 72, 96 and 120 hours. All plants were washed briefly in sterile water and frozen in liquid nitrogen and stored in the -80° freezer.

3.3.4 Long Carbohydrate Shift, Time-Course Four

Plant material used for this time course had been grown aseptically in continuous darkness for 3 weeks in Frick medium with a final concentration of either 2% glucose (Sigma G7528) or 2% galactose (Sigma G0750). The galactose used in this experiment contained a minimum of 99% galactose and contained < 0.01% glucose.

Plants were also switched from growing on 2% glucose Frick Medium lacking carbohydrate support. This was done to see how long it would take for the plant to stop utilizing any stored carbohydrates associated with growth in glucose. Plants were

sampled at 48, 72, 96 and 120 hours. All plants were washed briefly in sterile water and frozen in liquid nitrogen and stored in the -80° freezer.

3.3.5 RNA Extraction for QPCR

A 50 to 100 mg sample of previously frozen plants was ground into fine powder in liquid nitrogen using a tissue homogenization pestle and a 1.5ml snap cap tube. The powder was then processed using a Qiagen RNAeasy Plant Mini Kit (# 74903) per manufactures instructions.

3.3.6 cDNA Synthesis

Archiving of cDNA was done using the following reaction mixture (20ul per reaction). Using a 1.5ml snap-cap tube, add 1ul Oligo dT Primers (Promega C110A), 1ul, 10mM dNTP Mix 10mM (Promega U1515), (x)ul 200ng RNA and 8(x) ul H20. Incubate tube at 65° C for 5 min. Following the 5 min. incubation add the 10ul mix which consisted of 2 ul 5x buffer, 4ul 25 mM MgCl2,

2 ul DTT, 0.1M 1ul RNase Out 40U/ul (Invitrogen 18064-022). Incubate at 50° C for 50 min. Stop reaction by heating to 85° C for 5 min. Collect by brief centrifugation. To ensure that no traces of RNA remain in the cDNA 1ul RNase H (2U/ul) (Invitrogen) was added to the tube and incubated at 37° for 20 min.

3.3.7 Primer Probe Design

All primers for quantitative polymerase chain reaction (QPCR) were made using the ABI Primer Express Program version 2.0, using the sequences of the partial cDNA of SpGAL1 and SpHXK genes from *S. punctata*. The QPCR primer sets used were as follows:

Primer Pair One for SpGAL QPCR:

SpGALK-5F

5′ TTGTTGATGGAATAGTGCCCG 3′

SpGALK-70R

5′ TGGATGAGCAGACAAATGCG 3′

Primer Pair 2 for SpHXK QPCR:

SpHXK-134F

5′ CCAACTTCAGGGTACTGCGC 3′

SpHXK-199R

5′ CGAACTCCTGATTCCGAACG 3′

Primers were obtained from Operon (Valencia, CA).

3.3.6 QPCR

All QPCR reactions were performed in triplicate in a 384 well plate using an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA). Reaction mixtures of 20ul each were made as follows: 4ul Primer Set (1uM each primer), 4ul cDNA (35-40ng/ul), 2ul ROX reference dye (1:550, Stratagen # 600530-53), 10ul 2X SYBER Green QPCR Master Mix (Stratagen # 600548-51). After the addition of each component the plate was spun briefly to collect the liquid in the well bottom. QPCR parameters were set using the default cycle conditions of 95°C for 10 min followed by 40 cycles of 95° C for 30 sec and 60° C.

Figure 2A and 2B

Galactokinase Primer Design

Comparison of *Arabidopsis thaliana* Gal 1 (accession # AF024623) and *Glycine max* Gal1 (accession # AW 507994) sequences used for galactokinase primer design. Position of primers are in shaded areas.

Figure 2A Forward Galk1

Figure 2B Reverse Galk2

Figure 2A Forward Galk1

Accession Numbers	< Pos = 159
	AGGTGTGGATGTTGGTTCACCTGTTGGACTTGATGTTCTTGTTGATGGAATTGTTCCTACAG
	160 170 180 190 200 210 220
AF024623	AGGTGTGAATCTTGGTTCACCAGTTGGACTTGATGTGCTTGTTGATGGAATTGTTCCTACAG
AW507994	AGGAGTGGATGTTGGCAAACCTGTTGGACTTGAAGTTCTTGTTGATGGAACAGTGCCGACAG

Figure 2B Reverse Galk2

Accession Numbers	< Pos=459
	TGGGAGTTTTGTGATTGCTCATTCTTTTGCAGAGTCTCAGAAGGCTGTTACTGCTGCTACGA
	460 470 480 490 500 510 520
AF024623	AGGGAGTTTTGTAATTGCACATTCTCTTGCAGAGTCGCAGAAGGCGGTCACGGCTGCTAAGA
AW507994	TGGGACTTTTGTGATAGCTCATTCTTTGGCAGAGTCTCAAAAGGCTGTTACTGCTGCCACAA

Figure 3A and 3B

Hexokinase Primer Design

Alignment of conserved amino acid regions used for Hexokinase primer design tomato (*Lycopersicon esculentum*) Hxk2 (accession # LeHxk-AF208543), potato (*Solanum tuberosum*) Hxk1 (accession # StHXK1-X94302), *Arabidopsis thaliana* HXK1 (accession # AtHXK-U28214) and *Arabidopsis thaliana* HXK2 (accession # ATHXK2-U28215).

Figure 3A Forward Hex F

Figure 3B Reverse Hex R

Figure 3A Forward Hex F

Accession Numbers	< Pos =1
	PNSGEMVINMEWGNFRSS
	10
AF 208543	PNSGEMVINMEWGNFRSS
X94302	PNSGEMVINMEWGNFRSS
AF106068	PNSGEMVINMEWGNFRSS
U28214	PNSGEMVINMEWGNFRSS
U28215	PNSGEMVINMEWGNFRSS

Figure 3B Reverse Hex R

Accession Numbers	▫ Pos =15
	KLKQVADAMTVEMHA
	10
AF 208543	KLKQVADAMTVEMHA
X94302	KLKQVADAMTVEMHA
AF106068	KLKQVADAMTVEMHA
AF106068	KLKQVADAMTVEMHA
U28214	KLKQVADAMTVEMHA
U28215	KLKQVADAMTVEMHA

Chapter 4

Results

4.1 Amplification of hexokinase gene fragment from *Spirodela punctata*.

The SpHXK partial cDNA sequence retrieves 130 BLASTN hits from the GenBank database (May 2005). Its 612 bases match with confidence to cDNA sequences of *Malus x domestica* (95/107=88%), *Triticum aestivum* (74/80=92%), *Lactuca serriola* (75/83=90%), *Gossypium raimondi* (113/134=84%), *Nicotiana benthamiana* (68/74=91%), *Saccharun officinarum* (104/122=85%), *Oryza sativa* (77/87=88%), *Aegilops speltoides* (90/105=85%), *Secale cereale* (90/105=85%), *Panicum virgatum* (80/92=86%),etc. The UniGene link from the above *Triticum aestivum* match indicates a lengthy (200+ amino acids) correspondence with *Arabidopsis thaliana*, *Caenorhabdits elegans*, *Drosophila melanogaster*, *Homo sapiens*, *Mus musculus*, *Rattus norvegicus*, and *Saccharomyces cerevisiae* hexokinases.

The predicted amino acid sequence of SpHXK cDNA retrieves 113 BLASTP matches from the non-redundant GenBank CDS translations (May 2005). Its 204 residues match to predicted amino acid sequences of the hexokinases of ***Citrus sinensis*** (169/205=82%), ***Nicotiana tabacum*** (159/204=77%), ***Lycopersicon esculentum*** (158/204=77%), ***Solanum tuberosum*** (157/204=77%), ***Spinacia oleracea*** (160/205=78%), ***Arabidopsis thaliana*** (157/204=76%), etc. The NCBI Conserved Domain Search for the last match indicates confident matches to canonical hexokinases of a range of organisms.

We are led, therefore to believe that our partial, homologous, hexokinase clone SpHXK, would identify HXK, transcription in QPCR analysis.

4.2 Amplification of a galactokinase gene fragment from *Spirodela punctata*

The RT-PCR reaction produced a 314 basepair product which was cloned into pCR2.1.

The SpGAL1 partial cDNA sequence retrieves 118 BLASTN hits from the GenBank database (May 2005). Its 314 bases match with confidence to cDNA sequences of *Glycine Max* (307/314=97%), *Medicago truncatula* (287/314=97%), *Lotus japonicus* (222/237=93%), *Persea americana* (237/275=86%), *Royal Gala x domestica* (176/197=89%) *Vitis vinifera* (244/287=85%), *Citrus sinensis* (156/181=86%), *Lycopersicon esulentum* (235/286=82%), *Gossypium raimondii* (258/308=83%), etc. The UniGene link from the above *Glycine Max* match indicates a lengthy (210 amino acids) correspondence with *Arabidopsis thaliana*, *C.elegans*, *E. coli*, *H.sapiens* and *Saccharomyces cerevisiae* galactokinase GAL1.

The predicted amino acid sequence of SpGAL1 retrieves 240 BLASTP matches from the non-redundant, full-length GenBank CDS translations (May 2005). Its 105 residues match to predicted amino acid sequences of the GAL1 of *Pisum sativum* (99/104=94%), *Arabidopsis thaliana* (97/105=87%) and *Oryza sativa cv japonica* (85/105=80%), etc. The NCBI Conserved Domain Search

for this last match indicates confident matches to canonical galactokinases of a range of organisms, each have the **GLSSSAA** concensus signature sequence.

We are led, therefore to believe that our partial, homologous, galactokinase clone SpGAL1, would identify GAL1, transcription in QPCR analysis.

4.3 Carbohydrate Shift Time-Courses

4.3.1 Short Carbohydrate Shift, Time-Course One

The results of the QPCR expression study (Fig. 4, pg.66) did not support our hypothesis and do not resemble the catabolite repression model of ***S. cerevisiae***. Regardless of the carbon source chosen or the time of exposure the SpHXK mRNA levels remained up-regulated compared to the SpGAL1 mRNA expression levels.

We failed to see any GAL1 mRNA transcriptional expression at any of the time points.

4.3.2 Short Carbohydrate Shift, Time-Course Two

QPCR was used to test for mRNA expression levels of SpHXK mRNA and SpGAL1 mRNA. Regardless of the time points sampled 4, 8 or 24 hours or the choice of carbon sources, glucose or galactose, the SpHXK mRNA expression levels are up-regulated or equal to the steady-state levels (Fig.5, pg.67).

The SpGAL1 mRNA levels were never detected.

At the 4 hour time point we see an approximate 12 fold increase in expression over the HXK mRNA steady-state levels, when plants growing on galactose are switched to a 2% final concentration of glucose and galactose.

4.3.3 Long Carbohydrate Shift, Time-Course Three

QPCR was used to test for mRNA expression levels of SpHXK mRNA and SpGAL1 (Fig.6, pg.68). For time points 36, 48, 72 and 96 hours we see a steady increase in SpHXK mRNA expression levels compared to the steady-state levels.

At the 120 hour time point we see SpHXK mRNA expression levels begin to dip below the SpHXK mRNA steady-state levels.

For the first time at 96 hours we see an up-regulation of the SpGAL1 mRNA expression levels compared with the steady-state SpGAL1 mRNA expression levels. In no other QPCR test was SpGAl1 mRNA detected.

4.3.4 Carbohydrate Time-Course Four

QPCR was used to test for mRNA expression levels of SpHXK mRNA and SpGAL1. We see for time points 36, 48, 72, 96 and 120 hours SpHXK mRNA expression levels at steady-state levels (Fig. 7, pg.69). SpGAL1 was not detected.

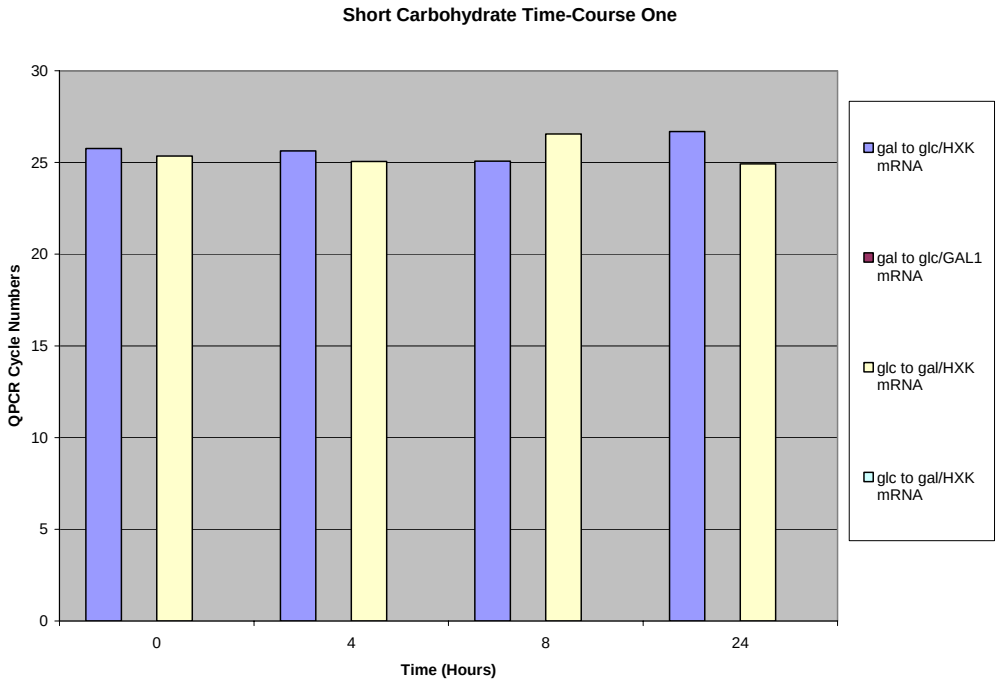


Figure 4: Short Carbohydrate Shift Time-Course One: HXK mRNA and GAL1 mRNA, are hexokinase and galactokinase QPCR products using primers of homologous partial cDNA clones respectively. The prefixes **gal** and **glc** indicate heterotrophic growth on either carbon source; **gal to glc** and **glc to gal** indicate the shift to the alternate carbon source at time 0.

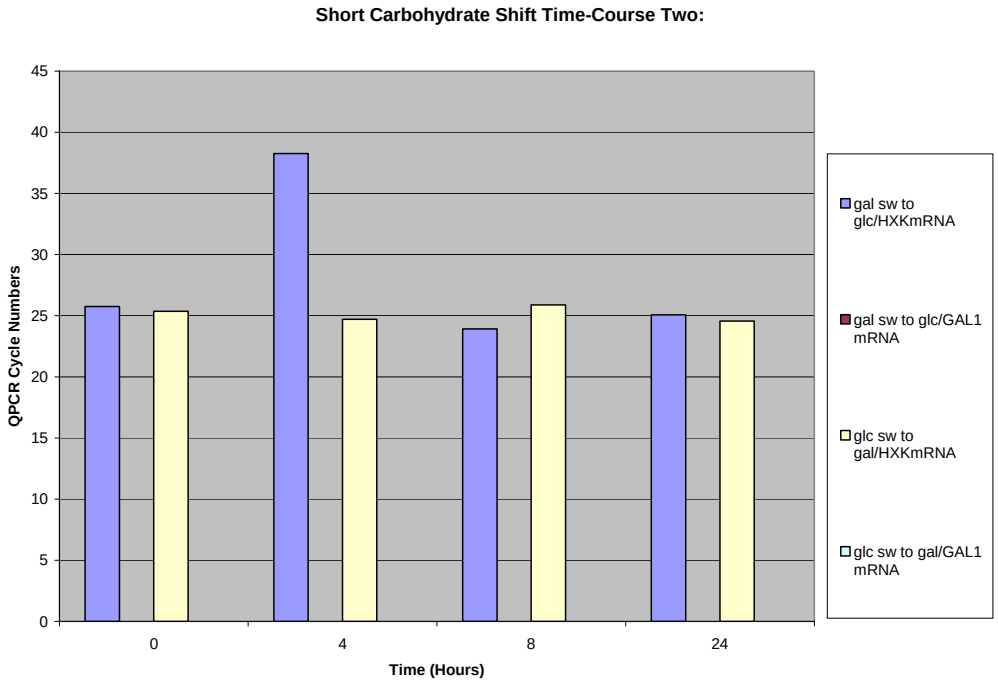


Figure 5: Short Carbohydrate Shift Time-Course Two: HXK mRNA and GAL1 mRNA, are hexokinase and galactokinase QPCR products using primers of homologous partial cDNA clones respectively. The prefixes **gal** and **glc** indicate heterotrophic growth on either carbon source; **gal to glc** and **glc to gal** indicate the shift to the alternate carbon source at time 0. The prefixes **gal sw to glc** and **glc sw to gal** indicate the shift from a single carbon source to a 2% final concentration of both carbon sources at time 0.

Figure 6: Long Carbohydrate Shift Time-Course Three: HXK mRNA and GAL1 mRNA, are hexokinase and galactokinase QPCR products using primers of homologous partial cDNA clones respectively. The prefixes **gal** and **glc** indicate heterotrophic growth on either carbon source; **gal to glc** and **glc to gal** indicate the shift to the alternate carbon source at time 0. The prefixes **gal sw to glc** and **glc sw to gal** indicate the shift from a single carbon source to a 2% final concentration of both carbon sources at time 0.

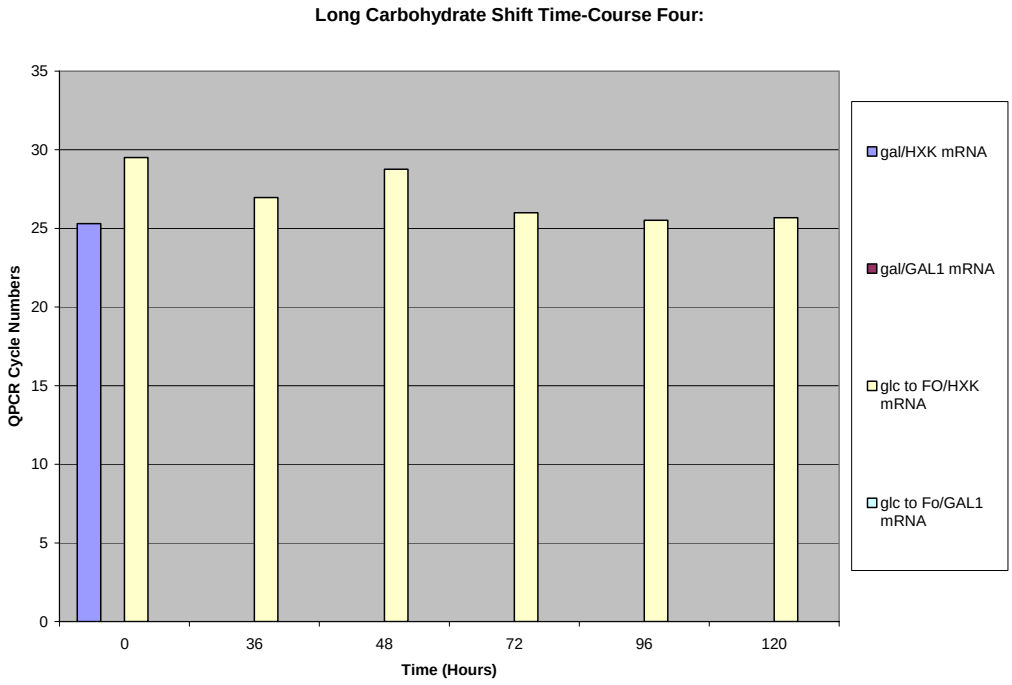


Figure 7: Long Carbohydrate Shift Time-Course Four: HXK mRNA and GAL1 mRNA, are hexokinase and galactokinase QPCR products using primers of homologous partial cDNA clones respectively. The prefixes **gal** and **glc** indicate heterotrophic growth on either carbon source; **gal to glc** and **glc to gal** indicate the shift to the alternate carbon source at time 0. The prefixes **gal sw to glc** and **glc sw to gal** indicate the shift from a single carbon source to a 2% final concentration of both carbon sources at time 0. The prefix glc to FO indicate the shift from glucose to Frick Medium without the addition of a carbon source.

Chapter 5

Discussion

5.1 Analysis of Gene Expression:

Saccharomyces cerevisiae prefers glucose as a carbon source, but can thrive on other carbon sources such as galactose. Glucose can affect enzyme levels by causing a decrease or down-regulation in the mRNA concentration levels for the utilization of alternative carbon sources such as galactose. Glucose also causes an increase or up-regulation of the enzyme levels that correspond to mRNA expression levels for this carbon source. This phenomenon is known as "catabolite repression" (Gancedo, 1998).

When *S. cerevisiae* is given galactose as a sole carbon source, the opposite "repression effect" can also be seen with an up-regulation of mRNA expression levels for the utilization of galactose and a down-regulation of mRNA expression levels for the utilization of glucose (Gancedo, 1998).

We hypothesized that *Spirodela punctata* would also show a classic catabolite repression model. This hypothesis was conceived on the fact that it has been reported that *Lemna minor* callus uses both the

Leloir and Isselbacher pathways to assimilate galactose and that galactokinase specific activity is up-regulated in the presence of galactose (Frick and Morley, 95). Due to the fact that *Spirodela* plants can utilize either glucose or galactose as the sole carbon source in continuous darkness we are postulating that it assimilates these hexoses in the same way as *Lemna* callus.

5.2 Short Carbohydrate Shift Time-Course One

Our first experiment was a short carbohydrate shift time-course (Fig.4, pg. 66). QPCR was used to test for mRNA transcriptional expression level changes of SpHXK mRNA and SpGAL1 mRNA. The two goals of this experiment were very simple. First, we wanted to see what the steady-state (0 hr) mRNA transcriptional expression levels were for SpHXK mRNA and SpGAL1 mRNA when *S.punctata* was utilizing either glucose or galactose as a sole carbon source for growth in continuous darkness. We wanted to know what this baseline level of expression is so that it could be

used as a reference point to determine if expression levels truly change in our other experiments.

Second, in order for us to say that *S. punctata* is utilizing both hexokinase and galactokinase as sugar sensors we must first show that it is displaying a catabolite repression model like *S. cerevisiae*.

S. punctata can utilize either glucose or galactose as the sole carbon source under continuous dark conditions. Since this is true the following should apply, SpHXK mRNA transcriptional expression levels should be up-regulated or equal to the steady state levels when *S. punctata* growing on galactose is switched to glucose, and we should see SpGal1 mRNA expression levels down-regulated compared to SpHXK mRNA expression levels. The inverse should hold true that SpGAL1 mRNA transcriptional expression levels should be up-regulated or equal to steady-state levels when plants growing on glucose are switched to galactose and the SpHXK mRNA levels should be down-regulated or equal to the SpGAL1 mRNA levels.

If this hypothesis holds true we could postulate that, *S. punctata* is using hexokinase and galactokinase as the sugar sensors because it is showing the results of a classic catabolite repression model.

The sample times of 4, 8 and 24 hrs were used for sampling (Fig. 4, pg.66). These points were chosen because it has been shown that yeast cells need time to adapt to new carbon sources. Yeast can adapt to glucose about 1.6 times faster than to galactose (Barnett, 2004). Dienert's experiments showed brewer's yeast can ferment galactose, but only after a period of induction with galactose. Yeast can ferment glucose without an induction period to glucose. Adaptation to both of these hexoses can occur without cell multiplication (Dienert, 1900).

The results of the QPCR expression study did not support our hypothesis and does not resemble the catabolite repression model of *S. cerevisiae*. Regardless of the carbon source chosen or the time of

exposure the SpHXK mRNA levels remained up-regulated compared to the SpGAL1 mRNA expression levels.

We failed to detect any SpGAL1 mRNA at any time. It is interesting to note that even though the QPCR primers used flanked the conserved galactokinase signature sequence of **GLSSSA,** SpGAL1 mRNA was never detected.

5.3 Short Carbohydrate Shift Time-Course Two

Glucose is the preferred carbon source of eukaryotic growth and metabolism (Rolland et al., 2002). *Spirodela punctata* does not seem to have this preference as it appears it can grow equally well on either glucose or galactose. The question proposed for this time course is the following: If given a choice of glucose or galactose, which one would *Spirodela punctata* prefer to utilize glucose or galactose after initial growth on either glucose or

galactose. Since this experiment was run concurrently with time-course one, the same zero/steady-state time points were used for comparisons.

At the 4, 8, and 24 hour time points the SpGAL1 mRNA expression is undetected, while the SpHXK mRNA expression remain up-regulated regardless of carbon source or exposure time. (Fig.5, pg. 67). At the 4 hr time point the switch from 2% galactose to 2% glucose there is about a 12 fold increase in the SpHXK mRNA expression level, compared to the SpHXK mRNA steady-state levels. This increase in expression levels may be due to *S.punctata* ability to quickly sense and begin using glucose or the inability of galactose to shut down the glucose sensing genes.

5.4 Long Carbohydrate Shift Time Course Three

This time course extended the sampling times due to the fact we were not seeing any expression level for SpGAL1 mRNA in the first two time courses and we felt that maybe there could be a greater lag time

involved between the switch of carbon source utilization then previously thought. We also decided to use a low glucose galactose (<0.01% glucose) as pure galactose is not commercially available. The galactose used in time courses 1 and 2 contained a minimum of 98% galactose and could contain up to 2% glucose in the galactose.

Using QPCR our zero time points attempted to measure SpHXK and SpGAL1 mRNA expression levels for plants utilizing glucose or galactose as a sole carbon source in continuous darkness. We wanted to try again to see if we could detect a steady state SpGAL1 level.

Our other experimental conditions also attempt to measure SpHXK and SpGAL1 mRNA expression levels, but only when plants are shifted from glucose to galactose as a sole carbon source. This was done in an effort to try and determine when SpGAL1 mRNA expression levels are up-regulated.

We see a steady increase in SpHXK mRNA expression levels (Fig. 6, pg. 68) starting at 36 hours through

96, then we see these levels start to drop-off at 120 hours compared to the steady state levels.

At the 96 hour time point for the first and only time we see an up-regulation of SpGAL1 mRNA expression levels, compared with the steady state SpGAl1 mRNA expression level. We failed to produce any QPCR signal for the steady state levels and must conclude that this is an anomalous event.

5.5 Carbohydrate Shift Time-Course Four

In this time course plants were grown on Frick Medium without the addition of hexose in continuous darkness. We wanted to see at what point growth in continuous darkness, would exhaust all starch reserves. This would be determined by the down-regulation of both SpHXK and SpGAL1 expression levels compared to the state-state levels.

Since this experiment was run concurrently with time-course 3 the same zero time points were used for comparison. *S.punctata* must have a tremendous capacity to store and utilize starch since it can proliferate and remain viable for 120 hours without the addition of any hexose (Fig. 7, pg. 69).

5.6 Final Thoughts

In QPCR, the baseline value is established during the initial cycles when there is only an insignificant change in the total sample fluorescence. As the cycles progress into the exponential phase the system will detect a cycle when the fluorescence detected is significantly higher than the baseline. It could be possible that the fluorescence never rises above this baseline level due to the fact there is such a small amount of PCR product accumulation, thus no signal is produced for SpGAL1 mRNA expression levels.

To amplify our partial SpGAL1 cDNA from *S. punctata* it required the use of mRNA for the template for the RT-PCR reaction instead of the use of

total RNA. The use of mRNA instead of total RNA may indicate that the mRNA transcript already exists in low abundance.

The lack QPCR signal could be due to the fact that the chemistry chosen for the QPCR detection of SpGAL1 mRNA was not optimal. Even though our QPCR designed primers were based on our SPGAL1 sequence and flanked the coding region for the galactokinase signature sequence **GLSSSAA** we still do not see SpGAL1 mRNA expression.

SYBR® Green 1 dye chemistry binds to non-specific dsDNA. Although this can be a sensitive assay it may not be specific enough for the detection of SpGAL1 due to the perceived low abundance of mRNA. When using SYBR Green 1 Dye for QPCR, the optimal amplicon length is less then 100 bp (Bustin, 2000). The Taqman system, which is template-specfic can detect amplicons as long as 400bp (Bustin, 1999). The Taqman assay (Perkin-Elmer. Applied Biosystems) requires the annealing of 3 different oligonucleotides to the cDNA, 2 of these oligonucleotides are template-specific and

define the ends of the amplicon (Bustin 2000). For SpGAL1 mRNA to be detected the use of a more specific chemistry and the ability to detect a larger amplicon, may be what is needed.

Chapter 6

Conclusions

1. A 612 base pair SpHXK gene fragment was cloned from *Spirodela punctata*.
2. A 314 base pair SpGAL1 gene fragment was cloned from *Spirodela punctata*.
3. QPCR shows that SpHXK mRNA transcriptional gene expression levels are always up-regulated

regardless of the carbon source or time of sampling.

4. An up-regulation of SpGAL1 mRNA is seen only at 96 hours post-transfer to galactose, as sole heterotrophic carbon source. This is an anomalous result. We did not see any up-regulation of SpGAL1 mRNA as it was not tested.

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