Isolation and partial characterisation of PHT1;5, a putative high affinity phosphate transporter from *Arabidopsis thaliana*

by

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SUMMARY

Inorganic Phosphate (Pi) is one of the key nutrients required by all living organisms on earth. This nutrient is of vital importance to higher plants but it is not readily available for uptake from the soil, implying constant stress on plants. During photosynthetic dark and light reactions, phosphate is a prerequisite for all reactions to occur and to ensure plant survival. This statement implies that a careful homeostatic control of this nutrient is necessary in order to maintain a balanced carbon flow in all sub-cellular plant compartments.

Phosphate limitation is a threat to plant survival and one way of addressing this nutritional hurdle is by feeding plants with fertilizer. This method of crop development and general plant maintenance by humans has a devastating effect on the environment, as phosphate causes eutrophication and various other consequences which are detrimental to animal life. Plants, however, are naturally equipped with Pi transporters which are activated conditionally depending on the external Pi availability. These transporters are present in most sub-cellular compartments and some of them have been identified and characterised, while others remain to be a prediction. If these transporters are characterised accordingly it might eventually mean that the use of fertilizers may no longer be necessary. In order to contribute to successful Pi-efficient crop development, a clearer understanding of P-dynamics in the soil and its recycling ability inside the plant itself is necessary.

During this study it was attempted to characterise a putative high affinity Pi transporter, PHT1;5, from *Arabidopsis thaliana* via a *Escherichia coli* and yeast heterologous expression system and its Km value predicted in order to verify/hypothesise whether it is a high or low affinity transporter. This transporter is expressed in leaves and could be a promising tool for future carbon partitioning studies during phosphate limitation.

OPSOMMING

Anorganiese fosfaat (Pi) word beskou as een van die belangrikste nutriente benodig vir alle lewe op aarde. Dit vervul ‘n hoof rol in talle noodsaaklike prosesse in hoër plante en is veral ‘n voorvereiste vir fotosintetiese reaksies om plaas te vind. In ‘n plant se natuurlike omgewing is anorganiese fosfaat nie geredelik bekskikbaar in grond nie en dus word daar vermoed dat plante onder konstante fosfaat stres gevind word. Omdat fosfaat so ‘n belangrike rol speel tydens fotosintese is dit noodsaaklik dat daar ‘n balans op sellulêre vlak gehandhaaf word, veral wat die verspreiding van koolhidrate tussen die verskillende kompartemente van die sel betref.

Plante se oorlewing word bedreig deur ‘n tekort aan fosfaat in die omgewing en die enigste onmiddelike oplossing daarvoor is deur die toediening van bemestingstowwe. Hierdie metode van landery ontwikkeling en algemene instandhouding van plante deur die mensdom het ’n baie negatiewe effek op die omgewing. ‘n Oormaat fosfaat lei tot eutrifikasie en het verkeie ander negatiewe nagevolge wat dodelik is vir die dierelewe. Plante beskik ook oor natuurlike interne fosfaat transporters om hierdie tekort te oorkom. Hierdie transporters word op grond van eksterne fosfaat beskikbaarheid ge-aktiveer of ge-deaktifeer. Die transporters is teenwoordig in meeste sub-sellulêre kompartemente en sommige is al ge-identifiseer en gekarakteriseer, terwyl ander slegs ‘n voorspelling bly.

Gedurende hierdie studie was ‘n poging aangewend om ‘n anorganiese fosfaat transporter van *Arabidopsis thaliana*, PHT1;5, te karakteriseer met behulp van mikro-organismes soos *Escherichia coli* en gis. Die poging het ingesluit om ‘n Km waarde vir hierdie transporter te voorspel en sodoende ‘n hipotese daar te stel van of dit hoë of lae affiniteit het vir fosfaat. Die transporter word groot en deels aangetref in blare en kan dus dien as ‘n belowende apparaat vir toekomstige koolhidraat uitruiling studies gedurende fosfaat tekort.

"Never measure the height of a mountain, until you have reached the top. Then you will see how low it was."

Dag Hammarskjld

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LIST OF ABBREVIATIONS

cDNA	Complementary DNA
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed Sequence Tag
gDNA	genomic DNA
GT	Glucose transporter
IPTG	isopropyl β-D-1-thiogalactopyranoside
L	Litre
M	Molar
MFS	Major facilitator superfamily
Mg	milli-gram
µg	micro-gram
µl	micro-litre
ml	milli-litre
µM	micro-molar
mM	milli-molar
MT	Maltose transporter
PCR	Polymerase Chain Reaction

Pi	Inorganic phosphate
Po	Organic phosphate
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SDS	Sodium dodecyl sulfate
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite repression
TE	Tris EDTA buffer
TEMED	Tetramethylethylenediamine
TM	Transmembrane
TMH	Transmembrane helix
TPT	Triose Phosphate Transporter
WT	Wild Type

CHAPTER 1

General Introduction

Inorganic Phosphate (Pi) plays a central role in most of the vital processes of higher plants prerequisite for photosynthesis to occur. It has been suggested to be the most important nutrient, next to nitrogen, but it is not readily available for uptake from the soil, implying constant stress on plants. Being the key factor during photosynthetic reactions, a careful homeostatic control of this nutrient is necessary in order to maintain a balanced carbon flow in all sub-cellular plant compartments.

Plant phosphate transporters are of extreme importance in the regulation of phosphate homeostasis on a whole plant level and are present in most sub-cellular compartments. The function of several phosphate transporters have been characterised, while others remain as *in silico* predictions. There are to date five families of transporters identified in *Arabidopsis thaliana* which are all localized to various compartments in the plant cell and present in different areas of the plant itself. Phosphate transporters can be categorised as high or low affinity based on their binding and transport capacity for phosphate at different concentrations. High-affinity transporters are activated during conditions of phosphate limitation and usually display a Km value of between 1-40 µM. Although many high-affinity phosphate transporters in plants have been putatively predicted, very little of them have been successfully characterised. Some of these plant phosphate transporters that show sequence similarities to high affinity transporters from *Saccharomyces cerevisiae* (Bun-Ya *et al*., 1991), *Neurospora crassa* (Versaw, 1995) and *Glomus versiforme* (Harrison and Van Buuren, 1995) have been characterised. These include LePT1 from *Lycopersicon esculentum* (Daram et al., 1998) with a Km value of 31 µM, StPT1 and StPT2 from *Solanum tuberosum* (Leggewie *et al*., 1997) displaying respective Km values of 280 µM and 130 µM (still in question about whether they are high or low affinity), PHT1;1 from *Hordeum vulgare* L. (Rae *et al*., 2003) with Km value of 9.06 µM. These transporters have proven to be very difficult to characterise because contradicting Km values are often found when these proteins are analysed in yeast or cultivated plant cells.

The molecular mechanisms that monitor phosphate availability and integrate the nutritional signal in plants are unknown but this machinery has been extensively studied in other organisms. In many instances the characteristics of these transporters have been explored through the use of well defined heterologous expression systems utilising yeast, bacteria, oocytes or insect cells (Daram et al., 1998; Guo, 2010). The phosphate uptake mechanisms for each of these systems have been studied to great extend and therefore created a platform on which to characterise similar high or low affinity phosphate transporters present in plants. Most of the plant transporters have been successfully characterised by means of heterologous expression systems and their biochemical properties exploited subsequently.

Phosphate limitation is a well known threat to plant survival and this nutritional hurdle is generally addressed by feeding plants with fertilizer, but plants are naturally equipped with high affinity Pi transporters which are activated during low Pi conditions. In the event of uncovering the biochemical properties of these transporters and understanding the mechanism behind phosphate sensing in plants, facilitates the development of genetically modified crops that constitutively express high-affinity Pi-transporters. This could serve as a possible approach to enhance plant survival in Pi-deprived soil, implying that the use of fertilizers may no longer be necessary.

During this study a putative high affinity Pi transporter from *Arabidopsis thaliana*, PHT1;5, was investigated by means of utilizing heterologous expression systems including the use of *E.coli* and yeast mutant strains. This transporter has been proposed to be expressed mainly in leaves and to be localized to the chloroplast, although evidence for the latter statement does exist, the strength of evidence is not particularly convincing. Exploiting the characteristics of this transporter could lead to a promising tool for future carbon partitioning studies during phosphate limitation. In the long-term, developing crops that are able to sustain growth during Pi limiting surrounds, this study might contribute greatly to the success of the agricultural industry.

CHAPTER 2

Literature Review

2.1 Importance of Phosphate to plants

Plants are dependent on a range of nutrients which are all important for survival and development. Phosphorus, classified as one of the most essential macronutrients, is available in two forms namely phosphate, the fully oxidised form, and orthophosphate (Pi) which is the assimilated form (Rausch and Bucher, 2002). Orthophosphate is utilised by plants to carry out various functions involved and required in many of the vital structural and regulatory processes in higher plants. These processes include growth, metabolism, energy transfer, enzyme activity regulation, photosynthesis and carbon partitioning (Walker and Sivak, 1986). This assimilated form of phosphorus is not readily available in soil for plant uptake due to it forming insoluble complexes with other minerals such as iron (Fe), aluminium (Al) and calcium (Ca) (Welp *et al*., 1983; Holford, 1997). It also adsorbs to the surfaces of clay minerals, soil particles and calcium and magnesium (Mg) carbonates (Rausch and Bucher, 2002). Not only is the solubility a limiting factor but it is present at very low micromolar concentrations (1-10 µM) in soil (Marschner, 1995), suggesting that there is a constant demand for Pi in plants. This implies that, unless Pi is supplied by fertilisers, plants are under phosphorus limitation in their natural ecosystem. In the schematic representation, Figure 1, it is shown how the phosphorus cycle is involved in the ecosystem between plant, microbes and soil interactions. It has no atmospheric connection like nitrogen (N), which means that phosphorus is not necessarily subject to undergo biological transformation.

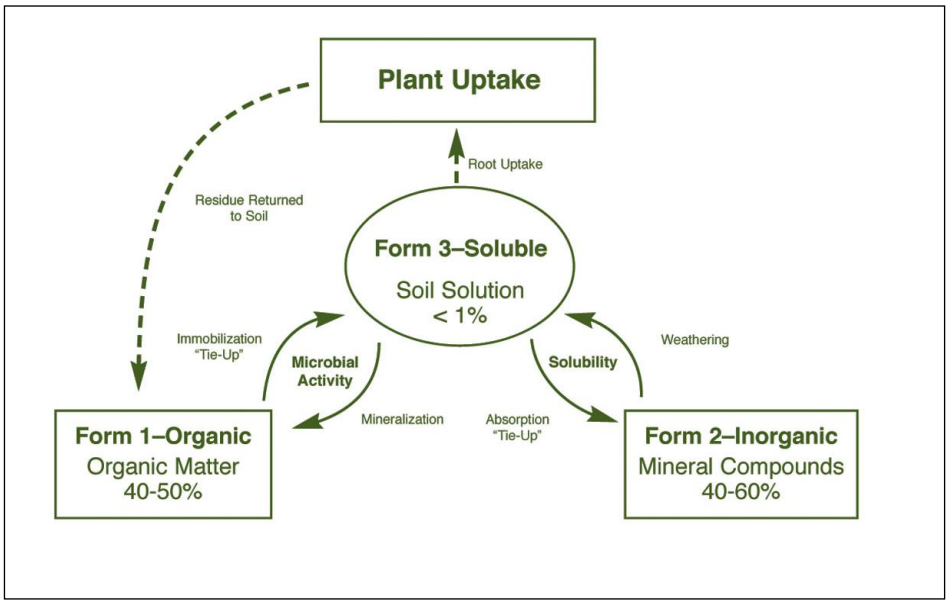

Figure 1: A simple schematic representation of how phosphorus is utilised and recycled in nature. Phosphate occurs in various forms, mainly organic (Po) (Form 1) and inorganic (Pi) (Form 2). Plants utilise the soluble inorganic phosphate (Form 3) present in soil in very low concentrations. Pi forms insoluble complexes with various minerals, often rendering it unavailable for plant uptake. Microbial activity is also a key process whereby Po is converted to Pi for plant use. Soluble Pi enters the plant through uptake by the root system. Once plants die off their remains can contribute to the recycling process of phosphate (http://sandhillsh3.com/sites-phosphorus-fertilizer-cannabis/)

Furthermore, phosphorus is structurally extremely important as it links together the genetic building blocks, DNA and RNA, enabling plants and all other living things to produce proteins and other compounds. These compounds are necessary for structural development, cell division and the development and repair of new tissue. Pi is also an important component of ATP which is the energy carrier in all living things. ATP is a three component structure which includes a phosphate chain, an adenine and a ribose sub-unit. The key element for ATP activity is the phosphate chain which then magnifies the important role that Pi plays as part of natures' energy source (Walker and Sivak, 1986).

2.2 Extracellular Phosphate transport

Although phosphate is abundant in soil, it is evident that the assimilated form (Pi) is not readily available for plant uptake. Due to this limiting nature of orthophosphate, plants often face endangerment in their natural ecosystems and had to develop various adaptation strategies to overcome this nutritional hurdle. The first adaptation that plants undergo is usually aimed at root architecture and an alteration in root-to-shoot development. The root system becomes shallow, primary root growth is inhibited, lateral roots elongate and root hairs are prone to become more dense (Peret *et al*., 2011). These changes in root structure and growth are proposed to be associated with plant hormones (Nacry *et al*., 2005), which suggest that phosphate limitation causes a systemic response rather than a localized response (Smith, 2002).

Phosphate is imported into plant cells, from the external soil surrounds, via the root system from where it is distributed accordingly to the aerial system (Smith, 2002). This import action from soil to root is driven by a rather lengthy diffusion mechanism which results into a phosphate depletion zone around the root system during phosphate limiting conditions (Smith, 2002). This zone, also known as the rhizosphere, is the main area for interaction between roots, nutrients and microbes (Shen *et al*., 2011). The morphological changes in the root architecture, as mentionad by Peret *et al*. (2011), are crucial steps in which plants are able to increase phosphate uptake and overcome a phosphate limitation in their soil surrounds. For example, topsoil foraging (Lynch and Brown, 2001), whereby lateral root growth prevails and primary root growth is temporarily halted (Ticconi and Abel, 2004), enable the plant to expand the immediate surface on which to search for favourable nutrient conditions, as phosphate becomes more limiting with greater depths (Vance *et al*., 2003). It is evidently also necessary for the root to increase its own surface area in order to maximise sufficient phosphate uptake. Root hairs account for up to 80% of the roots surface and are an important tool for plants to combat phosphate limitation by increasing its length and density (Lynch and Brown, 2001; Williamson *et al*., 2001; Ma *et al*., 2001; Ticconi and Abel, 2004). These actions are taken by plants which are unable to form association with microbes to assist in increasing surface area for phosphate uptake (Jungk, 2001).

Biochemical changes are also part of the external Pi-sensing mechanism which include the activation and secretion of certain enzymes such as phosphohydrolases. These enzymes are able to discharge Pi from organic matter which is the main component of phosphorus in soil. They also regulate the release of malate and citrate (organic acids) to chelate cations, such as Fe^{3+}, Ca^{2+} and Al^{3+}, which form insoluble salt and soil complexes, in order to facilitate the recruitment of inorganic phosphate (Lopez-Bucio *et al*., 2000; Raghothama, 2000; Abel *et al*., 2002; Vance *et al*., 2003).

Shen *et al.* illustrated an expanded view of how phosphate is recycled and distributed in the natural environment of the plant (Figure 2). Phosphate is present in soil in various forms, mainly organic and inorganic. Pi uptake by the plant seems to be a complex procedure and requires various individual processes. At first the plant is challenged with the reality of Pi absorbing to various minerals in the soil forming insoluble complexes (Figure 2: Soil processes). Furthermore, phosphate in soil is usually present in the organic (Po) form which is not useful to plants as they are only ably to use the assimilated form, inorganic phosphate (Pi). Po can be transformed to Pi by means of mineralization processes mediated by various microorganisms making it available for plant uptake. The rhizosphere is an essential area for the plant where Pi is made available to the plant through various processes (Figure 2: Rhizosphere processes). This is the zone where plant roots are able to interact with microorganisms that can assist in the uptake of Pi. Spatial and bioavailability of Pi greatly influence root growth and architecture and various soil properties. These impacts of Pi control the dynamics of phosphate in the rhizosphere area. The utilization, translocation and recycling processes of Pi inside the plant itself require even more complex and interlinked mechanisms (Figure 2: Plant processes). Phosphate is linked to all the key cross-talk processes in plants, suggesting that Pi dynamics inside the plant is systemic and not only based upon localised responses. In conclusion, plants have evolved their biochemical, morphological and physiological response mechanisms in order to transport Pi optimally and to effectively overcome limiting nutrient conditions.

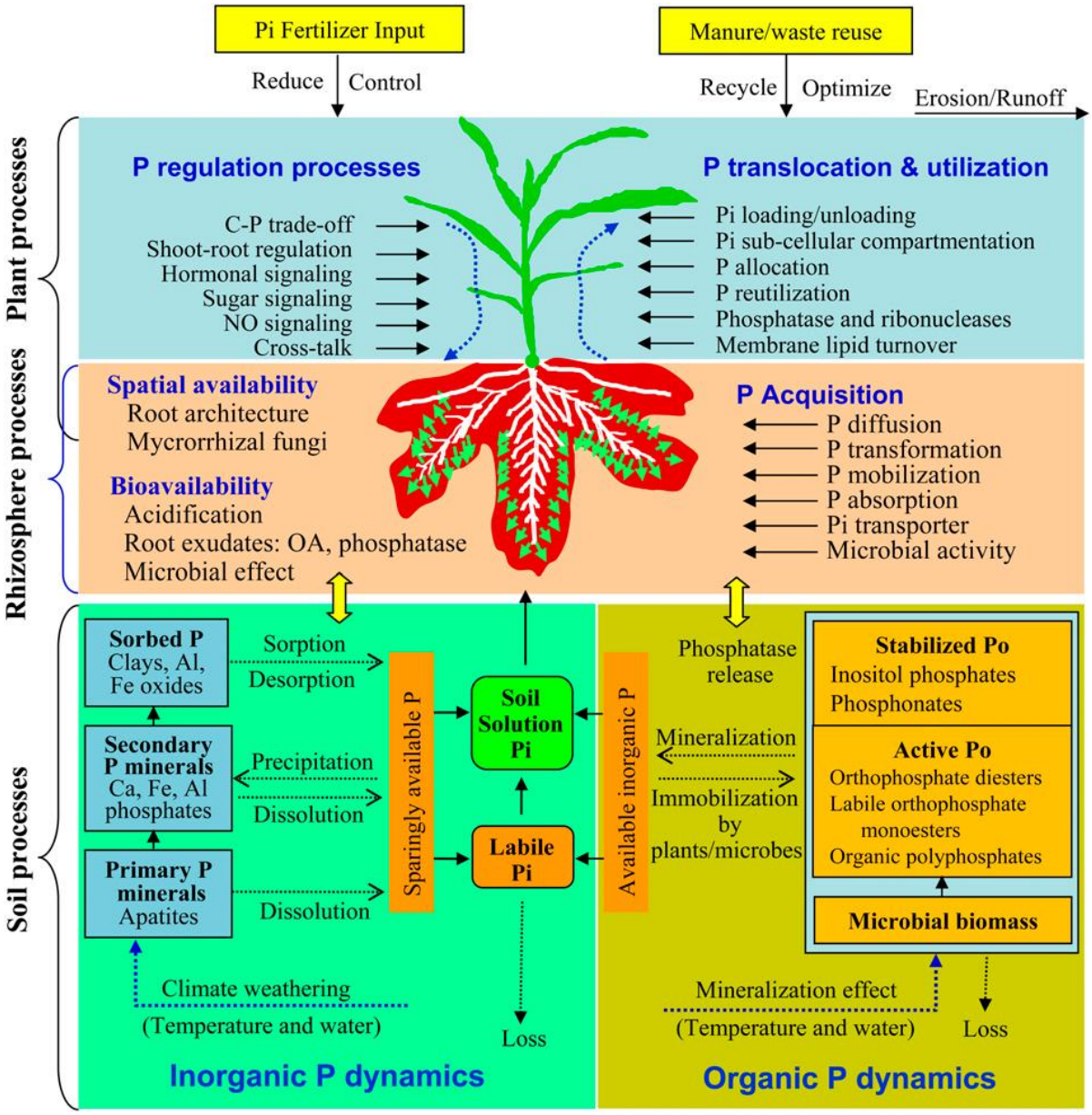

Figure 2: A schematic representation from Shen et al. (2011) indicating the recycling process of phosphorus - from soil to plant and vice versa

2.3 Intracellular Phosphate transport

Internally plants have developed and evolved in such a way to cope with the low availability of external Pi. These responses are aimed at the recycling of phosphate and include the use of high affinity transporter proteins activated under low Pi conditions (Smith *et al*., 2003). However, many of these transporters still remain only a prediction based on sequence similarity. Only a few of these transporters, as a result of bioinformatics and genetic or biochemical studies, have been identified in plants so far. Sequence similarity, based upon organisms such as yeast and *Neurospora crassa*, has made it possible to predict five families of transporters in *Arabidopsis thaliana* localized to different compartments of the plant cell (BunYa *et al*., 1991; Versaw and Metzenberg, 1995). These include: PHT1 (plasma membrane), PHT2 (plastid inner envelope), PHT3 (mitochondrial inner membrane), PHT4 (golgi membrane and plastid thylakoid membrane) and pPT (plastid inner envelope) (Rausch and Bucher, 2002; Knappe *et al*., 2003; Guo *et al*., 2008; Zwiegelaar, 2010). To date a number of transporters have been identified in *A. thaliana* (Table 1). These transporters, localised throughout the plant, might fulfil various functions such as phosphate transport.

During Pi limiting conditions cells are forced to remobilise and recycle their internal Pi which is stored in multiple compartments in the plant, including the vacuole. This recycling process includes the degradation of various macromolecules, co-factors and other intermediates which are distributed throughout the plant (Morcuende *et al*., 2007; Zwiegelaar, 2010). The main problem with this type of remobilisation is that the Pi stored in the vacuole is aimed at a long term solution and released at a very slow rate, too slow for the plant to gain sufficient amounts of Pi to undergo necessary photosynthetic reactions. This problem is addressed by activation of the high affinity Pi transporters. These transporters are mainly included in the Pht1 family which are not only subject to Pi uptake from the soil, but also proposed to be involved in the translocation of Pi from older and senescent leaves to the rest of the plant (Jeschke *et al*., 1997; Zwiegelaar, 2010). These high affinity transporters enable plants to overcome a phosphate limiting environment and are key elements required for the functional uptake mechanism for nutritionally challenged plants.

Goldstein *et al*., (1989) proposed an interesting concept for the recycling of phosphate in plants during limiting conditions. This hypothesis is based upon the well characterised yeast pho-regulon which encodes for Pi-acquisition enzymes and regulatory proteins. Coherently plants should also have the ability to control Pi-responsive genes on a physiological and genetic level, thus a 'pho-regulon' was suggested in analogy to microorganisms. This system includes the up-regulation of several enzymes and proteins which include a Pi-starvation inducible high-affinity Pi transporter, phosphatases with varying substrate specificities, ribonucleases, phosphoenolpyruvate carboxylases, pyrophosphate-dependent phosphofruktokinase and also several short open reading frames encoding for RNAs or polypeptides. The phosphatases and ribonucleases are believed to be involved in the degradation of extracellular nucleic acids and other organic compounds. They release phosphate for immediate use and supply phosphate on short term demand. Carboxylases and phosphofruktokinases are proposed to be involved in the Pi-recycling systems that bypass the Pi and adenylate requiring steps in glycolysis, thus permitting carbon metabolism to proceed in Pi-starved cells.

All of the phosphate transporters mediate an adequate supply of inorganic phosphate to the relevant subcellular compartments depending on the phosphate availability in the soil. Low Pi concentrations will activate a high affinity transporter which will compensate for the other systems unable to supply the chloroplast with Pi. This is supported by the fact that the function of the different transporter homologs seems to be conserved throughout the plant, microbial and animal kingdoms (Guo, 2008). These transporter proteins are essential to plants in order to maintain Pi levels in all subcellular compartments. This elucidates the fact that Pi is the key nutrient involved in the regulation of metabolic processes and that a careful homeostatic control is necessary to maintain the storage and redistribution of Pi in plant cells. However, there are more unexplained issues to this ability of plants to overcome Pi limiting conditions. The fact holds true that there are still remaining transporters that need to be identified and characterised before Pi homeostasis and transport can be completely grasped.

Table 1: Transporters included in the *Arabidopsis thaliana* families of transporters, their localisation and possible function

Gene name	Gene ID	Accession number	Tissue of expression	Assigned/Posiible function
PHT1;1	At5g43350	D86608	Root, Cotyledon, Shoot, Bud, Seed	H^+/Pi symporter
PHT1;2	At5g43370	AB000094	Root	H^+/Pi symporter
PHT1;3	At5g43360	AB000094	Root, Cotyledon, Leaf	H^+/Pi symporter
PHT1;4	At2g38940	AB016166	Root, Leaf, Flower, cultured cells	H^+/Pi symporter
PHT1;5	At2g32830	AL00397	Leaf, Flower, Bud	H^+/Pi symporter
PHT1;6	At5g43340	AB005747	Cotyledon, Phloem	H^+/Pi symporter
PHT1;7	At3g54700	ATT5N23	Root, Flower	H^+/Pi symporter
PHT1;8	At1g20860	-	Root	H^+/Pi symporter
PHT1;9	At1g76430	AAF20242	Root	H^+/Pi symporter
PHT2;1	At3g26570	AJ302645	Aerial organs	Chloroplast Pi symporter
PHT3;1	At5g14040	BAB08283	-	Mitochondrial Pi transporter
PHT3;2	At3g48850	-	-	Mitochondrial Pi transporter
PHT3;3	At2g17270	-	-	Mitochondrial Pi transporter
PHT4;1	At2g29650	NM_128519	Photosynthetic tissues	Plastid thylakoid membrane transporter
PHT4;2	At2g38060	NM_129362	Root	Plastid Pi transporter
PHT4;3	At3g46980	NM_114565	Leaf phloem	Plastid Pi transporter
PHT4;4	At4g00370	NM_116261	Photosynthetic tissues	Plastid Pi transporter
PHT4;5	At5g20380	NM_122045	Leaf phloem	Plastid Pi transporter
PHT4;6	At5g44370	NM_123804	Ubiguitous	Pi transporter in Golgi membrane
AtTPT	At5g46110	AAC83815	-	Triose-phosphate/Pi translocator
AtPPT	At5g33320	AAB40646	-	Phosphoenolpyruvate/Pi translocator
AtGPT1	At5g54800	AAL15310	-	Glucose-6-phosphate/Pi translocator
AtGPT2	At1g61800	-	-	Glucose-6-phosphate/Pi translocator
AtXPT	At5g17640	AF209211	Flower, Leaf, Shoot, Root	Pentose phosphate/Pi translocator

2.4 Transporters included in the Pht1 family

Several putative Pi transporters have been identified and categorised to the Pht1 family according to the *Arabidopsis* EST database cDNA sequence similarities. The sequence similarties were based upon genes from species with characterised Pi transporters such as PHO84 from *Saccharomyces cerevisiae* (Bun-Ya *et al*., 1991), PHO5 from *Neurospora crassa* (Versaw, 1995) and GvPT from *Glomus versiforme* (Harrison and Van Buuren, 1995). The first genes to be cloned and putatively identified as Pi transporters were isolated from *Solanum tubersum* and *Arabidopsis* cDNA libraries (Muchhal *et al*., 1996; Leggewie *et al*., 1997; Mitsukawa *et al*., 1997) or by RT-PCR (Smith *et al*., 1997) and were subsequently assigned to this family. Since then the number of genes from several other plant species have been identified as Pi transporters including genes from *Lycopersicon esculentum* (Daram *et al*., 1998; Liu *et al*., 1998a) and *Medicago truncatula* (Liu *et al*., 1998b) which have been successfully characterised. These transporters showed an increase in transcript levels during Pi limiting conditions and were for this reason assigned to the Pht1 family of high affinity transporters.

The Pht1 family of transporters are all H^+-coupled transporters that facilitate the movement of phosphate across the plasma membrane against an electrochemical gradient (Marschner, 1995). The fully sequenced genome of *A. thaliana* has made it possible to identify 9 members of transporters included in the Pht1 family. These can be accessed by a gene search on the Arabidopsis Information Resource website: http://arabidopsis.org/info/genefamily/genefamily.html. All of these genes exhibit a high similarity in sequence and encode for high affinity inorganic phosphate transporters. Some of the properties of these transporters have been characterised (Muchhal *et al*., 1996; Smith *et al*., 1997; Okumura *et al*., 1998; Mudge *et al*., 2001) and Km values seems to be generally in the micro molar range.

Eight out of the nine members in Arabidopsis were shown to be expressed predominantly in roots. In the roots these transporters mediate the movement of Pi against a steep concentration gradient due to an internal plant Pi concentration which is approximately 1000 to 100 000 times higher than that of the soil (Shin *et al*., 2004). Although the

expression patterns of these transporters have been identified, their function regarding Pi movement inside the plant has yet to be determined (Karthikeyan *et al*., 2002; Mudge *et al*., 2002). This family of transporter proteins indicate 75%-85% similarities and were assigned to the Major Facilitator Superfamily (MFS) class of transporters (Pao *et al*., 1998).

The MFS contains membrane transporters and is one of the largest families of transporter classes next to the ATP-binding cassette superfamily (ABC). Both these families are found to be universal and present within most, if not all, living things (Pao *et al*., 1998). Transporters included in the MFS exhibit similar protein characteristics and generally display 12 to 14 putative or assigned transmembrane domains which are targeted to the plasma membrane (Pao *et al*., 1998). The MFS functions as a secondary carrier of small soluble molecules as a response against chemiosmotic ion gradients. It consists of seventeen sub-families including the Phosphate: H^+ superfamily (PHS) which is of importance to this study. The PHS family of transporters are conserved throughout the fungi, yeast and plant kingdoms but does not occur in bacteria, animals or other eukaryotes (Pao *et al*., 1998). Members of this family exhibit very similar sequence identity and proteins are generally in the range of 518 to 587 amino acid residues in length with 11 residues being conserved throughout.

2.5 PHT1;5

During this study a member, PHT1;5, of one of the families was investigated. This high affinity Pi transporter, expressed during Pi limiting conditions, is predicted to be localized to the chloroplast plasma membrane and is proposed to transport Pi as result of an electrochemical gradient brought about by H^+-ATPase (BunYa *et al*., 1991; Versaw and Metzenberg, 1995). This gradient permits transport to occur through a counter exchange H^+/Pi symport mechanism, similar to the model seen in Figure 3.

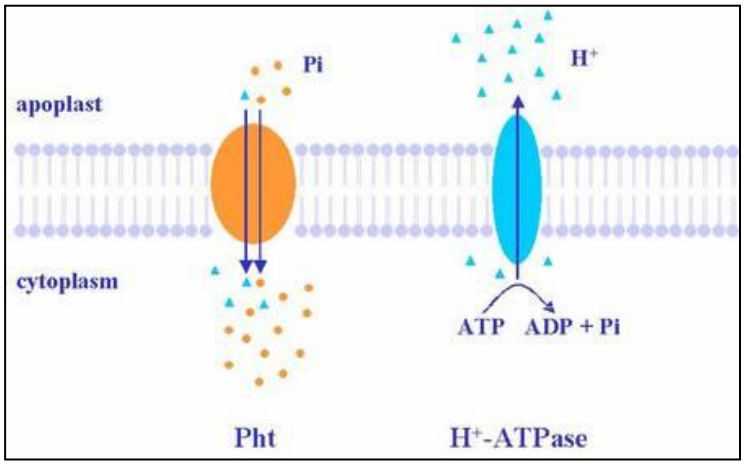

Figure 3: Movement of Pi across the plasma membrane (Illustration by Poirier and Bucher, 2002)

The H^+/Pi class of Pi transporters have been shown to be expressed predominantly in roots, although PHT1;5 and few other were proposed to be expressed in shoots (Okumura *et al*., 1998). Limited knowledge is available about the transport of Pi in the shoots where transport occurs between subcellular compartments such as chloroplasts/plastids, vacuoles and mitochondria. Various questions still remain about a large proportion of these transporters, such as the biochemical parameters (Km values, pH optimum, ion specificity), the biological role of these Pi transporters and the regulation of the genes during plant developmental stages and environmental responses.

A previous study by Mudge *et al*. (2002) indicated with reporter gene fusions that *PHT*1;5 is expressed in young plant tissue, cotyledons, and in older senescing leaves. This study suggested that PHT1;5 functions as a transporter which remobilize Pi from older to younger tissue, however their experiments were conducted during conditions of sufficient Pi availability. Zwiegelaar (2010) investigated expression patterns of *PHT*1;5 during conditions of Pi limitation which indicated that it is strongly expressed in leaves under these circumstances. Subcellular localization of PHT1;5 was subsequently determined to be targeted to the plastid. Zwiegelaar (2010) also demonstrated that double the amount of Pi was present in *Arabidopsis* WT chloroplast fractions as opposed to *PHT*1;5 T-DNA insertion mutants. These results suggested that this transporter acts as an importer of Pi into the chloroplasts during limiting conditions in order for photosynthesis to continue as normal.

A recent study by Nagarajan *et al*. (2011) utilised reverse genetics to determine the role of PHT1;5 in Pi mobilization, acquisition and its relation to ethylene signalling. They demonstrated that PHT1;5 is expressed in seedling cotyledons and hypocotyls, senescent leaves, floral buds and Pi-starved roots. It was suggested that PHT1;5 performs a similar role as the PHT1 transporters found in rice (OsPht1;2 and OsPht1;6) and barley (HvPht1;6) which are known for their function of Pi mobilization (Rae *et al*., 2003; Ai *et al*., 2009). In their study they indicated that PHT1;5 regulates the amount of Pi in shoot tissues by remobilizing Pi between root and shoot. It was indicated that PHT1;5 is localised in phloem of older leaves, sustaining the suggestion of its role in Pi remobilization. This highlights the important role of PHT1;5 during Pi homeostasis. Nagarajan *et al*. also investigated the interaction between ethylene signalling pathways and the activation of PHT1;5. They were able to demonstrate that over expression of this gene in Arabidopsis leads to premature senescence. It was revealed that Pi limiting conditions and senescence require similar intracellular actions which involve the recycling of Pi by releasing it from organic forms of phosphate (Zwiegelaar,2010). This evidence supports the hypothesis that there is a link between senescence, ethylene signalling and the activation of Pi transporters, but requires further investigation.

2.6 Phosphate, photosynthesis and PHT1;5 – the theory

The chloroplast harbours the photosynthetic network which is the main process that enables all plants to produce the necessary components for life, namely, starch and sucrose. Carbon assimilation during photosynthetic reactions requires orthophosphate (Pi) which is derived from ATP. This process is carried out in the chloroplast stroma and can be represented by the following equation, indicating Pi as key factor (Sivak and Walker, 1985):

$$3CO_2 + 6H_2O + Pi + h\nu \longrightarrow \text{triose phosphate} + 3H_2O + 3O_2$$

This simple equation indicates the plastidial import and export of metabolites through the phosphate translocater (Heldt and Rapley, 1970). It emphasizes the fact that the chloroplast

imports Pi and exports triose phosphate (Heldt and Rapley, 1970; Walker, 1976; Edwards and Walker, 1983) in a one-to-one ratio, thus the total phosphate inside the chloroplast is regulated to maintain a constant concentration. This illuminates the statement that Pi homeostasis plays a pivotal role in photosynthesis.

Carbon partitioning during the light and dark photosynthetic reactions occur mainly as result of Pi which regulate metabolic pools in the plant cell. The fluxes of Pi and carbon are highly coupled and regulated processes. The distribution of carbon between metabolites and different compartments of the cell remains to be the topic of on-going research. Increasing current knowledge of carbon flow can be achieved by investigation of two important areas. Firstly, the transport proteins on the plastid envelope membrane should be identified and characterised. Secondly, the rate of transport and its regulating mechanisms should be exploited. The first Pi transporter to be cloned and characterised was TPT which mediates a controlled passive counter exchange between Pi and triose-P photosynthetic end products during adequate Pi supply (Flugge *et al*., 1989). TPT is a low affinity transporter and therefore has restricted activity during Pi limiting conditions. This would lead to the inhibition of photosynthesis due to a lack of Pi in the chloroplast stroma. This statement is supported by the fact that TPT is the only low affinty transporter which directs the influx of Pi into the chloroplast (Guo, 2008).

It is thus predicted that the activation of the high affinity transporter, PHT1;5, is an alternative route of Pi supply during phosphate depletion. As a result of the poor functionality of TPT during phosphate limitation, alternative routes for carbon export should also exist in order to feed non-photosynthetic tissues and to maintain metabolic pools. It has been proposed that starch is broken down to maltose and glucose which are exported to the cytosol (Figure 4,), via GT (Weber *et al.,* 2000) and MT (Niittyla *et al*., 2004) where it is converted into sucrose. This statement is supported by the fact that mutants of these transporters are coupled to impaired starch degradation (Weber *et al*., 2000).

The vacuole is the main storage pool of Pi and comprises of 85-95% of the total Pi content of the plant (Rausch and Bucher, 2002). The effect that Pi has on photosynthesis and carbon partitioning are greatly influenced by the vacuole, cytoplasm and chloroplast Pi concentration (Rausch and Bucher, 2002; Zwiegelaar, 2010). During times of sufficient Pi supply, the concentration of Pi in the cytoplasm is around 60-80 µM (Pratt *et al*., 2009) and ensures for optimal photosynthetic conditions. However, during times of Pi limitation this concentration can decrease to levels below 15 µM (Pratt *et al*., 2009), affecting the rate of photosynthesis and carbon turnover. The immediate train of thought is that the vacuole would compensate for this lack of Pi by releasing its stored contents, but this holds untrue as the efflux rate of Pi from the vacuole is extremely slow (Martinoia *et al.,* 1986). The vacuole would therefore not attend to an immediate solution for metabolic availability of Pi but rather serve as a long-term solution. These statements were investigated during various studies where vacuoles were isolated to investigate their Pi uptake and release mechanisms. This statement was proven in a study done by Loughman *et al.,* (1989). They indicated that, when plants are fed sequestering agents like mannose, the Pi concentration inside the cytosol rapidly decreased while the concentration in the vacuole remains largely unchanged. The mechanisms by which the vacuole transports Pi is still fairly uncharacterised and further studies need to be conducted (Rausch and Bucher, 2002).

In order to ensure survival, plants had to develop other mechanisms by which to supply the chloroplast with Pi during limiting conditions. The short term solutions mainly involve the activation of high affinity transporters and transport of Pi across the chloroplast membrane (Zwiegelaar, 2010). Figure 4 illustrates the proposed alternative route of Pi supply to the chloroplast during Pi deprivation which is transported via the high affinity PHT1;5 for ATP production and photosynthesis to continue as normal. Photosynthesis generates an H^+ gradient (Figure 4: 1) to ensure ATP synthesis. This process requires a generous amount of Pi in order to prevent the H^+ gradient from increasing to such a level where photosynthesis is aborted. The low affinity transporter, TPT, ensures that the chloroplast is supplied with Pi (Figure 4: 2) by exchanging it for triose-P during phosphate-rich periods. During times of Pi limitation, when TPT is unable to function, it is proposed that the high affinity transporter, PHT1;5, is utilised to supply the chloroplast with Pi (Figure 4: 3). PHT1;5 is thought to be localized on the chloroplast membrane and has been shown to be up-regulated during times

of Pi limiting conditions. It is therefore hypothesised that PHT1;5 compensates for the lack of activity in the TPT transporter during these periods. Plants need to regulate cell metabolism (Figure 4: 4) which is achieved through starch turnover. This process contributes to Pi recycling through ATP hydrolysis, ensuring that photosynthetic reactions are maintained. During starch turnover, intermediates like glucose and maltose are exported to the cytosol (Figure 4: 5) to sustain cell metabolism (Weber *et al*., 2000; Niittyla *et al*., 2004). The enzyme β-amylase (Figure 4: 6) has increased activity during Pi limitation, suggesting its involvement in carbon flow by increasing the rate at which glycans are converted to hexose phosphate. This function contributes to the alternative route of carbon flow and Pi supply to chloroplast during limiting conditions but this pathway of degradation is still to a great extend unknown(Zwiegelaar 2010).

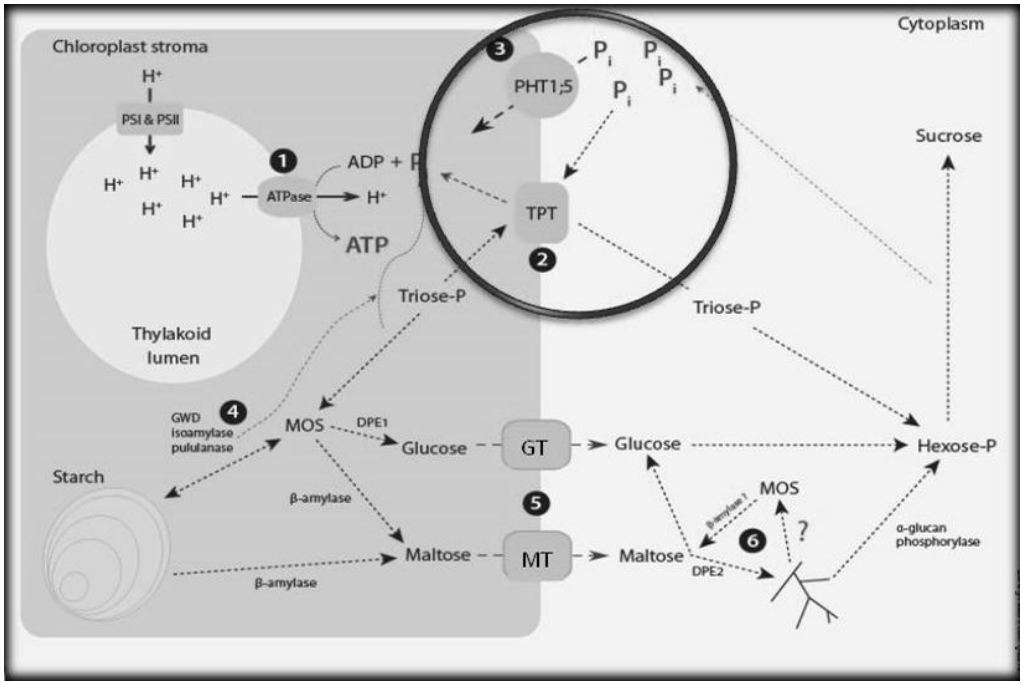

Figure 4: Illustration of an alternative route for carbon export during Pi limiting conditions (Illustration adapted from Zwiegelaar, 2010)

2.7 Heterologous expression systems

Heterologous expression systems are simplified platforms on which to characterise plant genes and to produce proteins on large scale outside their natural environment. These platforms usually include the use of bacteria, yeast, oocytes or insect cells which are all well defined systems (Wanner and Latterell, 1980; Rao and Torriani, 1990; Martinez and Persson, 1998). This enables researchers to perform uptake studies using heterologous expression systems, hence providing a platform on which to analyse and characterise similar transporters present in plants and/or animals.

Heterologous expression systems have made it possible to exploit the properties of similar transporters present in plants. *Saccharomyces cerevisiae* and *Pichia pistoris* have been utilised on previous occasions with great success (Leggewie *et al*., 1997; Smith *et al*., 1997; Daram *et al*., 1998; Guo, 2008). *Escherichia coli* systems have also been successfully utilised (Haferkamp *et al*., 2002; Pavon *et al*., 2008) where various NA+/Pi transporters have been exploited. The main heterologous expression systems used to characterise plant membrane transporters are therefore yeast and *E.coli*.

E.coli has been a favourite amongst the choice of well established heterologous expression systems (Frommer and Ninnemann, 1995). It displays several advantages above other systems such as simplicity to work with, rapid growth rate, high yield of protein production and relative low expense. Generally, genetic and biochemical information about this prokaryote is readily and extensively available, various strains and mutants are easy to come by and all commercially available vectors are compatible for *E.coli* transformation. The main disadvantage for this choice of heterologous expression system is the fact that *E.coli* is not able to execute post-translational modification, often a crucial step required in the folding of some recombinant proteins (Frommer and Ninnemann, 1995). Apart from the incorrect folding, another disadvantage is that the protein might be toxic to the bacterial cell. In some cases however, membrane proteins have been expressed successfully utilising this system for example the light-harvesting chlorophyll binding protein from pea (Kuhlbrandt and

Wang, 1991). Occasionally plant membrane transporters are characterised with this simple expression system and it has been proven to successfully conduct complementation assays and direct flux measurements (Kim *et al*., 1998; Uozomi *et al*., 1998). During a study conducted in 2008 by Pavon and colleagues, the *Arabidopsis* anion transporter ANTR1 was functionally characterised utilising *E.coli* as choice of expression system (Pavon *et al*., 2008). They demonstrated via radioactive analysis that this transporter functions as a Na^+-dependant Pi transporter and that it is localised on the thylakoid membrane of the chloroplast.

The eukaryotic system of heterologous expression, yeast, is frequently utilised to functionally express proteins from higher organisms such as plants. This ability displayed by yeast is further enhanced because eukaryotes share biochemical, genetic and molecular characteristics. Probably the main advantage of using yeast as a heterologous expression system is its ability to perform post-translational modification, thus producing functional recombinant proteins at a relatively rapid growth rate (Bassham *et al*., 2000). The shortcomings of this system are the extremely low yield of proteins produced and the possibility that the secreted proteins might be hyperglycosylated due to the stress level of yeast cells when expressing a foreign protein (Yesilirmak and Sayers, 2009). Frommer and Ninnemann (1995) investigated the ability of *S. cerevisiae* null mutants to be complemented by similar plant genes. They screened cDNA libraries from *Arabidopsis* and it was established that this heterologous expression system is suitable for studying the functional and kinetic properties of plant transporters (Dreyer *et al*., 1999). Previous investigations successfully exploited the electrophysiological characteristics of membrane transporters through complementation of mutant *S. cerevisiae* strains. For example KAT1 (Bertl *et al*., 1995), AtPT1 and AtPT2 from *Arabidopsis* (Muchhal *et al*., 1996), LePT1 and LePT2 from tomato (Daram *et al*,. 1998) and various transporters from plant species such as barley and grapevine (Santa-Maria *et al*., 1997; Hayes *et al*., 2007). On the downside, many of these transporters were only able to complement the mutant yeast strains but this expression system could not be utilised to investigate functional and kinetic properties.

Heterologous expression is a valuable system for investigation of biochemical and functional properties of genes from different organisms, especially where the genome sequence is not available. The disadvantages of each system must also be taken into consideration when protein structure and function need to be determined, due to the fact that when foreign genes are expressed, the recombinant protein might not fold correctly and might be mis-localised. Strategies to overcome misfolding have been developed. These include the use of other host organisms, expression of genes in the periplasm and the use of tags. Mis-localization is still an unresolved issue due to the possibility of the recombinant protein compensating for the absent protein of the host strain by replacing its function (Bassham *et al*., 2000). This increases the possibility for biased and unreliable results.

2.8 Aims and objectives

The aim of this study was to characterise the PHT1;5 Pi transporter from *Arabidopsis thaliana* and to exploit its characteristics for further investigations regarding alternative downstream carbon partitioning pathways under Pi limiting conditions.

There were three objectives set out for this study. The first objective was to investigate *E.coli* and yeast as heterelogous protein expression system. The second objective was to determine whether PHT1;5 is a high affinity transporter and characterise it by utilising a heterologous expression systems to approximate the Km value. This *E.coli* platform included the use of four mutant *E.coli* strains which all individually contained a gene deletion in the *pst* operon (*pst*A, *pst*B, *pst*C or *pst*S). A fifth strain mutated in all Pi uptake systems was also utilised. The yeast strain PAM2, mutated in both high and low affinity phosphate uptake systems, was also used. These strains were to be complemented by the addition of the *PHT*1;5 gene by functional expression when phosphate starvation conditions were initiated. The third objective was to characterise the protein *in silico* and with protein induction and expression *in vivo*.

CHAPTER 3

In Silico analysis of *PHT*1;5

3.1 Introduction

The high affinity transporter, PHT1;5, is part of the Pht1 family of transporters which are involved in the acquisition and transport of inorganic phosphate (Pi). They all display a protein size of approximately 58 kDa and a length of about 520 – 550 amino acids. Being part of the multi facilitator superfamily (MFS) it is suggested, but should not be assumed, that similar characteristics apply to all of the family members (Pao *et al*., 1998). These characteristics include 12 membrane spanning domains (MSD) which are arranged in a 6+6 configuration with a long hydrophilic loop extended between transmembranes six and seven. The transmembranes usually display an intracellular orientation for the hydrophilic loop as well as the N and C terminals. *In silico* analysis was done on PHT1;5 in order to determine whether it forms part of the predicted family of transporters and to indicate its expression pattern within the plant.

3.2 Materials en methods

TargetP (Emanuelsson *et al*., 2000), ChloroP (Emanuelsson *et al*., 1999) and PCLR (Schein *et al*., 2001) was utilised to predict the location of PHT1;5 on a subcellular level. The programs on the ARAMEMNON Web site (http://aramemnon.uni-koeln.de/) PRODIV-THMM, SOSUI, OCTOPUS and Mobyl were used to analyse membrane topology (Schwacke *et al*., 2003; Viklund *et al*., 2004; Gomi *et al*., 2004) and the expression profile of the protein. ClustalW was used to perform alignments of various sequences (Altschul *et al*., 1997).

3.3 Results and discussion

3.3.1 Analysis of the *Arabidopsis* PHT1;5 protein structure and membrane topology

The 542-amino acid sequence of PHT1;5 protein (UniProtKB Q8GYF4) from *Arabidopsis thaliana* was investigated on the ChloroP 1.1 (Emanuelsson *et al*., 1999) server for possible evidence that it could be localised to the chloroplast. The output obtained during prediction indicated very weak evidence for this transporter to be localised to the chloroplast. It displayed a weak probability score of 0.438 which is indicative of how certain the network is of an existing transit peptide (0 being least and 1 being most certain) and it was therefore predicted not to contain a signal peptide. Although the network suggested the absence of a transit peptide, it still determines the possible length of a putative chloroplast transit peptide, should one exist. This putative transit peptide was predicted to be 69 amino acids in length. The results concerning the transit peptide was further analysed on the TargetP 1.1 server (Emanuelsson *et al*., 1999) which predicts sub-cellular locations of proteins. TargetP displayed similar concerns with regards to PHT1;5 being localised to the chloroplast, with a cTP (chloroplast transit peptide) score of 0.015. TargetP predicted that this protein has another location (0.784) in the plant cell, excluding the mitochondria (score of 0.075) or the possibility of it being a secretion protein (score of 0.435). A final attempt to decide whether the protein is localised to the chloroplast was done on the PCLR server which indicated that PHT1;5 is non-chloroplast targeting. These results are contradictory to literature evidence. Although not conclusive, it should be taken into consideration that these programs merely predict the results and are not really probabilities, but only predicted as being most likely according to the software algorithms. The full length protein has 542 amino acid residues (Table 2), starting with a methionine, and the molecular mass for the full-length protein is 59 kDa.

Table 2: Amino acid composition of the PHT1;5 protein.

Number of amino acids contained within PHT1;5	Amino Acid Residue
20	Argenine
18	Asparagine
19	Aspartic acid
20	Glutamic acid
50	Glycine
23	Lysine
20	Proline
36	Serine

A hydrophobicity plot was analysed for the PHT1;5 protein to determine whether it has more polar or non-polar amino acid residues. A more positive value is indicative of hydrophobic amino acid residues at a specific position of the protein. Evidently the more negative the value, the more hydrophilic the residues. These plots are generally useful for predicting transmembrane alpha-helices that are included in membrane proteins because the amino acid residues are screened from start to end, values are plotted onto the scale as such, and each value indicates the association of the amino acid with the phospholipid membrane. The positive and negative values change accordingly and respectively denote whether or not the residue is attracted to or repelled by phospholipid membrane. Analysis of PHT1;5 with the Mobyl (von Heijne, 1992; Claros and von Heijne, 1994) software indicated that the protein is mostly hydrophobic (Figure 5) with a general positive score of 0.33, indicating that the protein is highly associated with the phospholipid membrane. It can therefore be assumed that PHT1;5 is a membrane bound protein.

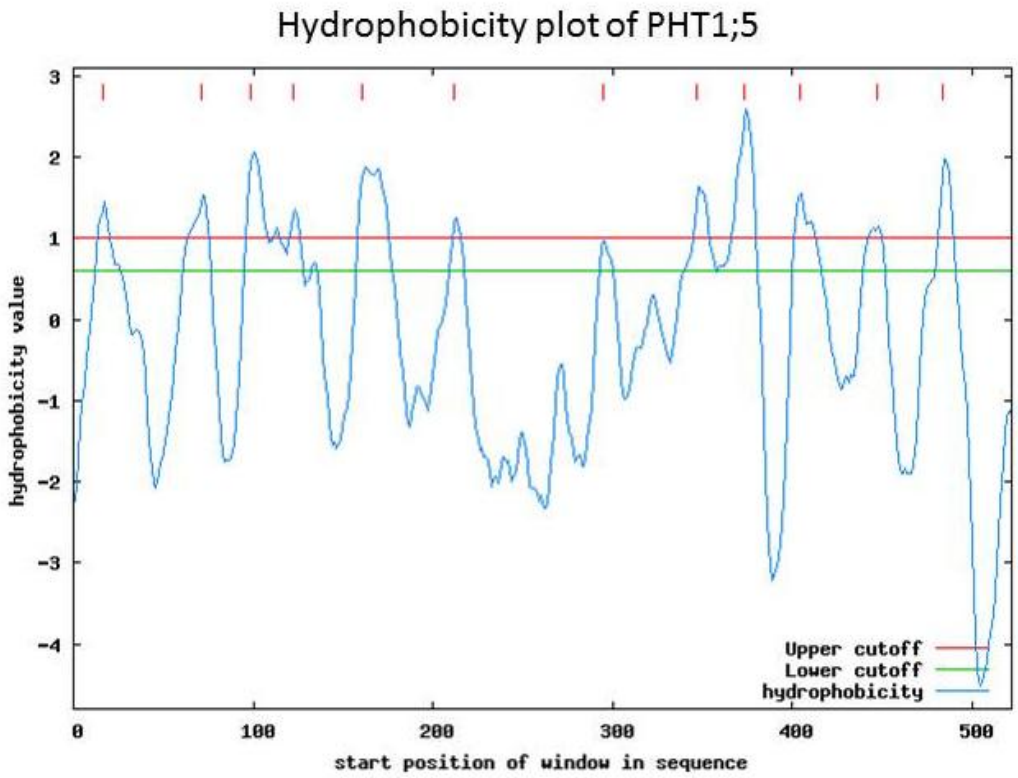

Figure 5: Hydrophobicity plot of PHT1;5 as predicted by Mobyl. Above hydrophobic regions are predicted transmembrane helices. Higher values indicate hydrophobicity, thus more positive values denotes the likeliness of the transmembrane to be hydrophobic and predicted to be associated with a membrane. In the plot it is clearly demonstrated that each transmembrane has a positive value and it can therefore be said that PHT1;5 is a membrane bound protein

The membrane topology of the PHT1;5 protein was determined with various software including TMHMM (Moller *et al*., 2001), SOSUI (Gomi *et al*., 2004), OCTOPUS(Marques *et al*., 2003) and Mobyl (von Heijne, 1992; Claros and von Heijne, 1994). These programs allow for the prediction of possible transmembrane alpha helices and also the location of the membrane loops. TMHMM predicted that approximately 247 amino acid residues are included in 10 putative transmembrane helices in the protein (Figure 6). The N- and C-terminals are predicted to have an “in” (for example, “in” the stroma) orientation which is an indication of it being a membrane bound protein. It was also found that there is a possibility that the N-terminal signal sequence may exist which contradicts previously generated data, but opens the possibility that it may be assigned to a specific organelle such as the chloroplast. The results obtained from this prediction are based on a hidden Markov

model, a statistics based program developed to make probabilistic models for linear sequences.

In addition, SOSUI software also predicted that there are 10 transmembrane helices but that the protein contains no signal peptide. Other sets of topology programs were utilised to analyse the protein but there is large variability in the outcomes between them. Some programs such as OCTOPUS and Mobyle predict the protein to have 12 membrane spanning domains (Figure 7) and that one of these proteins is only putative. Results gathered the various software are contradictory and might be due to fact that the protein contains a large number of proline and glycine residues as well as numerous charged residues which might interfere with the program algorithms. Similar problems were detected with the analysis of the ANTR1 (Pavon *et al*., 2008) and ATP/ADP carrier (Thuswaldner *et al*., 2007). In this study it is proposed, based upon assumption, that the protein contains 12 transmembrane helices simply because PHT1;5 belongs to the major facilitator superfamily (Lemieux, 2007) of H^+-transporters.

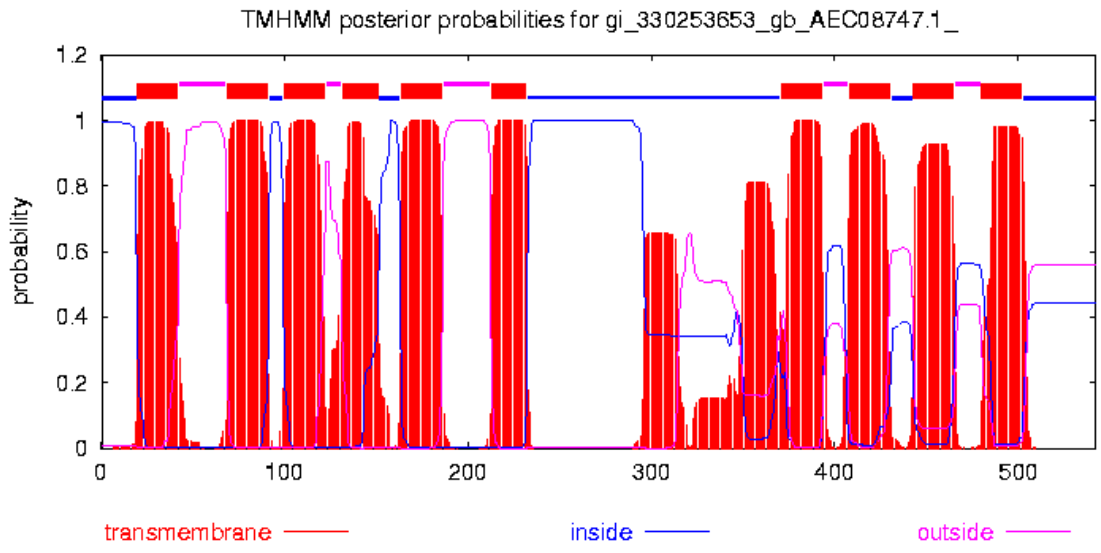

Figure 6: TMHMM software prediction of 10 putative transmembrane helices of the PHT1;5 protein

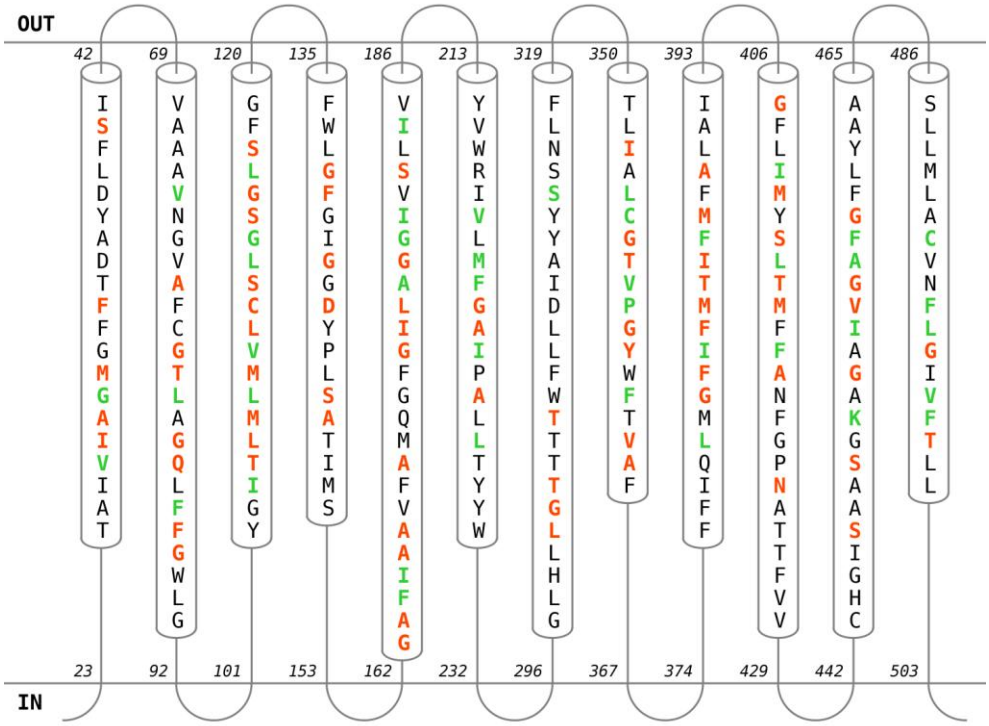

Figure 7: OCTOPUS prediction of 12 transmembrane helices in PHT1;5

3.3.2 Expression profile of *PHT*1;5

Previous studies (Zwiegelaar, 2010; Nagarajan *et al*., 2011) had proposed that the *PHT*1;5 is being expressed in the aerial parts of the plant. In order to observe this phenomenon *in silico,* analysis was performed with GeneCAT (Mutwil *et al*., 2008) software (Figure 8) which revealed that these findings hold true. GeneCAT indicated that the gene is highly expressed in senescing leaves, flowers, pollen and seed. These results have been confirmed in the Arabidopsis eFP browser and a similar expression profile was observed (Figure 9; Figure 10). The results obtained via the software indicate that the expression of *PHT*1;5 is highest in senescent leaves, implicating its possible role during phosphate remobilization from older to younger tissue during phosphate limitation. These findings lead to the speculation that PHT1;5 not only functions as an importer of Pi but it also implicates the possibility that it may function as an exporter during times of phosphate depletion, hence playing an important role in maintaining Pi homeostasis in sub-cellular compartments.

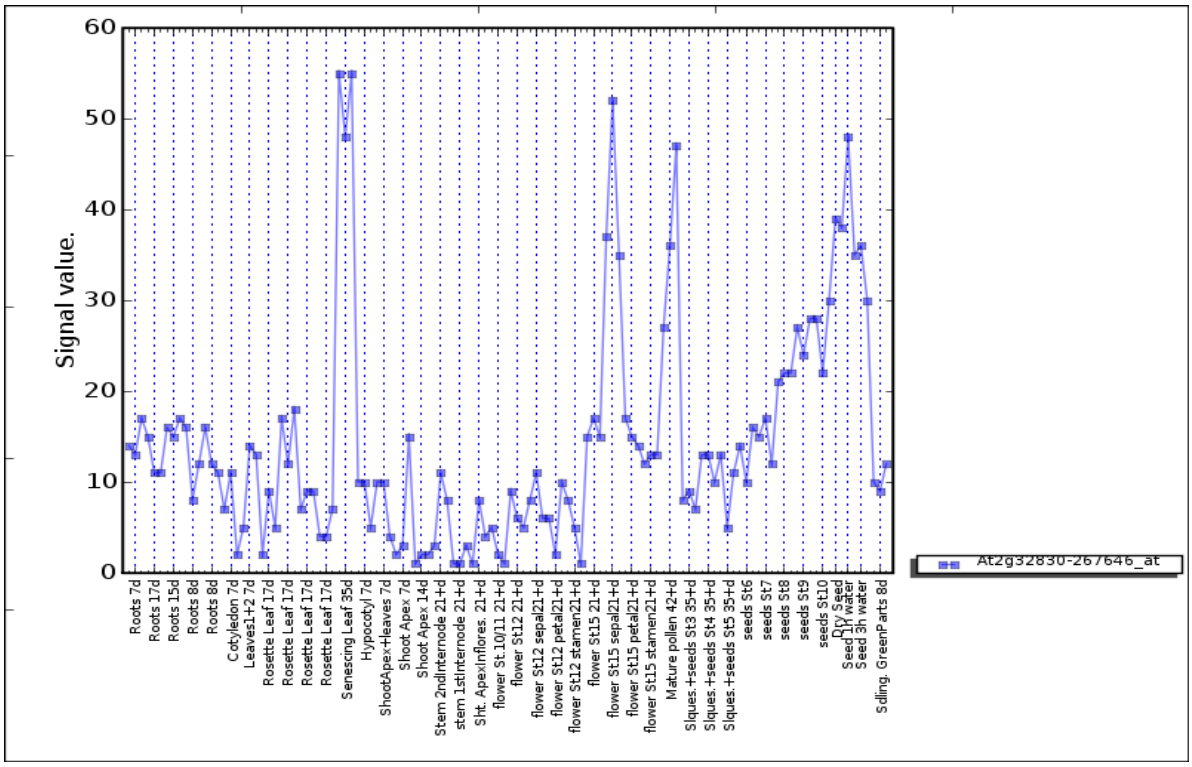

Figure 8: Expression profile of *PHT*1;5 as predicted by GeneCAT,indicating that the gene is highly expressed in senescent leaves

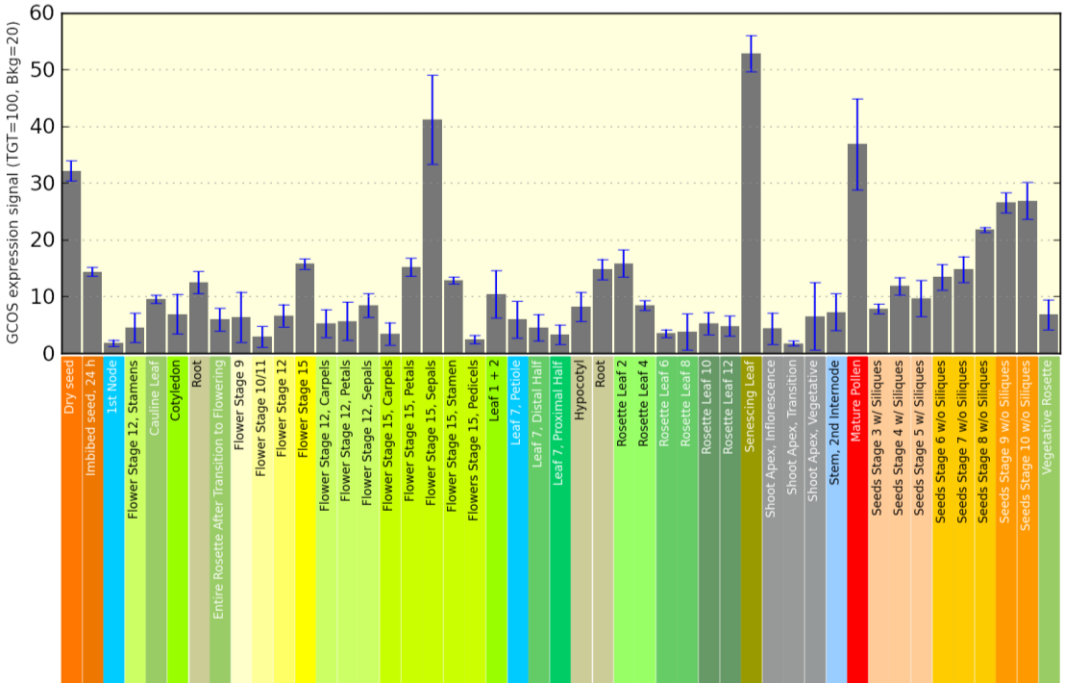

Figure 9: Expression profile of *PHT*1;5 as predicted by the Arabidopsis eFP browser

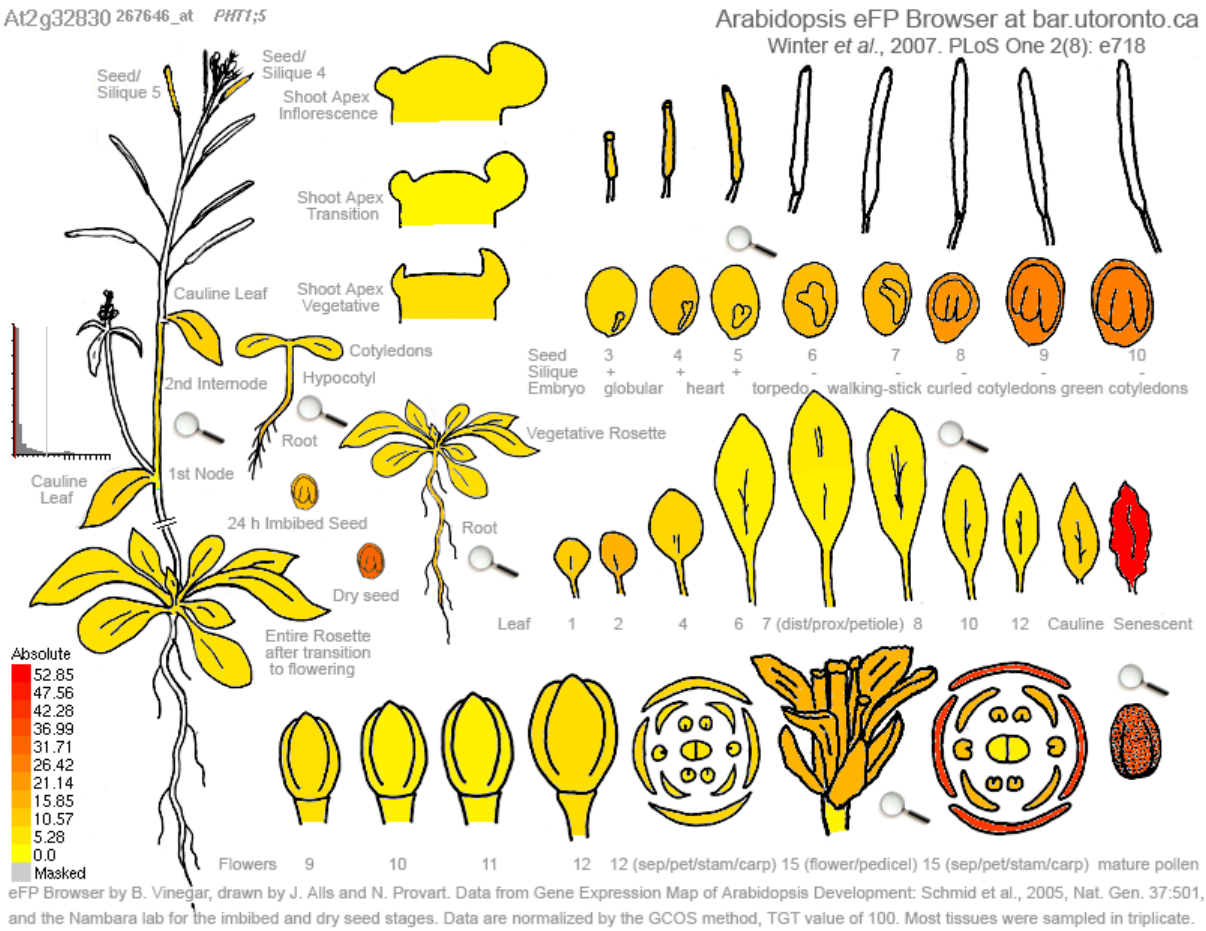

Figure 10: Development map of *PHT*1;5 as predicted by Arabidopsis eFP browser, confirming results obtained from the GeneCAT investigation. This expression profile indicates that *PHT1*;5 is highly expressed in senescent leaves. The map indicates different stages of plant development and predicts where the expression of the gene is highest; Yellow = no expression, Orange = moderate expression, Red = high level of expression

3.3.3 Sequence analysis of PHT1;5

Sequence alignments were done between proteins included in the Pht1 family of transporters and also between PHT1;5 and some known high-affinity transporter proteins from other species, such as PHO84 from yeast, LePT1 from tomato, PHO5 from *Neurospora crassa* and GvPT from *Glomus versiforme* (Figure 12). It was found that that sequences in the Pht1 family are highly similar and that is conserved region between amino acid positions 142-156: GIGGDYPLSATIMSE. When the sequence was further analysed it became evident that the transporter is part of the Major Facilitator superfamily (MFS) with conserved amino

acid residues, present amongst Pi/H^+-transporters from various species (Figure 12). No sequence homology was found to transporters in *E.coli* or other known plant chloroplast transporters.

```
PHO84           MSSVNKDTIHVAERSLHKEHLTEGGNMAFHNHLNDFAHIEDPLERRRLALESIDDEGFGW
PHO5            ---------------MSTPQKTAGGNNAYHNFYNDFLHIKDPNERRRLALAEVDRAPFGW
PHT1_5          ------------------------------------------MAKKGKEVLNALDAAKTQM
LePT1           -------------------------------------------MANDLQVLNALDVAKTQL
                                                          .   .*  :*

PHO84           QQVKTISIAGVGFLTDSYDIFAINLGITMMSYVYWHGSMPGPSQTLLKVSTSVG------
PHO5            YHVRAVAVAGVGFFTDSYDIFTVSLLTLMLGIVYFQAKARCLQPPTQPSSSQHR------
PHT1_5          YHFTAIVIAGMGFFTDAYDLFSISLVTKLLGRIYYHVDSSKKPGTLPPNVAAAVNGVAFC
LePT1           YHFTAIVIAGMGFFTDAYDLFCISMVTKLLGRLYYHHDGALKPGSLPPNVSAAVNGVAFC
                 :. :: :**:**:**:**:* :.:   ::. :*:: .      .     :

PHO84           -TVIGQFGFGTLADIVGRKRIYGMELIIMIVCTILQTTVAHSPAINFVAHSPAINFVAVL
PHO5            -PARSLG--------RSASAPLLMSLVVRACTDWNCCSSSLPLSLRPWLRVTFHQHHRYH
PHT1_5          GTLAGQLFFGWLGDKLGRKKVYGITLMLMVLCSLGSGLSFG---------HSANGVMATL
LePT1           GTLAGQLFFGWLGDKMGRKKVYGMTLMIMVICSIASGLSFG---------HTPKGVMTTL
                 .  .           . .    : *::                      .

PHO84           TFYRIVMGIGIGGDYPLSSIITSEFATTKWRGAIMGAVFANQAWGQISGGIIALILVAAY
PHO5            YLLACSYGGRYRWRLSSFQYITSEFATTKWRGAMMGAVFAMQGLGQLAAAFVMLFVTLGF
PHT1_5          CFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGIVSLIVSSTF
LePT1           CFFRFWLGFGIGGDYPLSATIMSEYANKKTRGAFIAAVFAMQGFGILAGGMVAIIVSAAF
                 :     *       .    * **:*..* ***::.**** *. * ::..:: :::   :

PHO84           KGELEYANSGAECDARCQKACDQMWRILIGLGTVLGLACLYFRLTIPESPRYQLDVNAKL
PHO5            KKSLEAAPTLASCTGDCAVAVDKMWRTVIGVGAVPGCIALYYRLTIPETPRYTFDVKRDV

PHT1_5          DHAFKAPTYEVDPVGSTVPQADYVWRIVLMFGAIPALLTYYWRMKMPETARYTALVARNT
LePT1           KGAFPAPAYEVDAIGSTVPQADFVWRIILMFGAIPAGLTYYWRMKMPETARYTALVAKNL
                .  :  .   ..  .      * :** :: .*:: .    *:*:.:**:.**   *  .

PHO84           ELAAAAQEQDGEKKIHDTSDEDMAINGLERASTAVESLDNHPPKASFKDFCRHFGQWKYG
PHO5            EQASDDIEAFKTGKPKGQPDE-----ATRIVAKQEAEKEMEIPKASWGDFFRHYSKRKNA
PHT1_5          KQAASDMSKVLQVDLIAEEEA-------------QSNSNSSNPNFTFGLFTREFAR-RHG
LePT1           KQAANDMSKVLQVEIEAEPEK-------------VTAISEAKGANDFGLFTKEFLR-RHG
                : *:   .     .     :                .       :  * :.: : : .

PHO84           KILLGTAGYWFTLDVAFYGLSLNSAVILQTIGYAGS---KNVYKKLYDTAVGNLILICAG
PHO5            MLLAGTALSWCFLDIAYYGVSLNNATILNVIGYSTTGA-KNTYEILYNTAVGNLIIVLAG
PHT1_5          LHLLGTTTTWFLLDIAYYSSNLFQKDIYTAIGWIPAAETMNAIHEVFTVSKAQTLIALCG
LePT1           LHLLGTASTWFLLDIAFYSQNLFQKDIFSAIGWIPPAQTMNALEEVYKIARAQTLIALCS
                   * **:  *  **:*:*. .* .  *  .**:  .    *. . ::  : .: ::  ..

PHO84           SLPGYWVSVFTVDIIGRKPIQLAGFIILTALFCVIGFAYHKLGD----HGLLALYVICQF
PHO5            AVPGYWVTVFTVDTVGRKPIQFMGFGILTILFVVMGFAYKHLSP----HALLAIFVLAQF
PHT1_5          TVPGYWFTVAFIDILGRFFIQLMGFIFMTIFMFALAIPYDHWRHRENRIGFLIMYSLTMF
LePT1           TVPGYWFTVAFIDKIGRFAIQLMGFFFMTVFMFALAIPYHHWTLKDHRIGFVVMYSFTFF
                ::****.:*  :* :**  **: ** ::* :: .:.:.*.:        .:: :: :  *

PHO84           FQNFGPNTTTFIVPGECFPTRYRSTAHGISAASGKVGAIIAQT---ALGTLIDHNCARDG
PHO5            FFNFGPNATTFIVPGEVFPTRYRSTSHGLSAAMGKIGSIIGQG---AIAPLRTRGAVKGG
PHT1_5          FANFGPNATTFVVPAEIFPARLRSTCHGISAASGKAGAIVGAFGFLYAAQSSDSEKTDAG
LePT1           FANFGPNATTFVVPAEIFPARLRSTCHGISAAAGKAGAMVGAFGFLYAAQPTDPTKTDAG
                * *****:***:**.* **:* ***.**:*** ** *:::.      .       .  *

PHO84           KPTNCWLPHVMEIFALFMLLGIFTTLLIPETKRKTLEEINELYHDEIDPATLNFRNKNND
PHO5            NPN-PWMNHVLEIYALFMLLGVGTTFLIPETKRKTLEELSGEFDMSGEEEAQRDTTLTEH
PHT1_5          YPPGIGVRNSLLMLACVNFLGIVFTLLVPESKGKSLEEISREDEEQSGGDTVVEMTVANS
LePT1           YPPGIGVRNSLIVLGCVNFLGMLFTFLVPESNGKSLEDLSRENE---GEEETVAEIRATS
                 *    : : : : . . :**:  *:*:**:: *:**::.   .

PHO84           IESSSPSQLQHEA
PHO5            KTEAPTSSAAVNA
PHT1_5          GRKVPV-------
LePT1           GRTVPV-------
                    .
```

Figure 11: Sequence alignment between PHT1;5 and high-affinity transporters from other species. Fully conserved residues are indicated with "*", highly conserved areas with ":" and weakly conserved areas with "."

3.3.4 Gene IDs

GenBank accession number for PHT1;5 is NP_180842.1 and may be accessed on the NCBI database (http://www.ncbi.nlm.nih.gov/) for reference. TAIR gene ID is At2g32830 and may be accessed on the Arabidopsis website www.arabidopsis.org/. MIPS gene ID for access on the Arabiopsis genome database http://mips.helmholtz-muenchen.de/plant/athal/ is At2g32830. To access the protein sequence on the Universal Protein Resource (UniProt) website www.uniprot.org/, the the protein ID Q8GYF4 should be used.

3.4 Concluding remarks

In silico analysis of the PHT1;5 protein sequence revealed ambiguous results and it should be kept in mind that the various software programs have different algorithms to which they calculate their values. ChloroP and TargetP indicated that PHT1;5 is not strongly predicted to be localized to the chloroplast, although there is an indication of a possible putative transit peptide 69 amino acids in length. It can be speculated that the PHT1;5 protein is targeted to the inner membrane of the chloroplast as is the case for transporters containing transit peptides.

The hydrophathy plot suggests that PHT1;5 is an integral membrane protein. Transmembrane helices (TMHs) were predicted with a number of programs indicating that there are either 10 (TMHMM and SOSUI software) or 12 (OCTOPUS and Mobyle software) membrane spanning domains. Although TMHMM is by far the most trusted program to predict these membrane helices, it can be assumed that transporters from the same superfamily display similar characteristics in their structure and it is therefore proposed that PHT1;5 should have 12 TMHs.

Expression analysis revealed that the gene is expressed strongest in senescent leaves. This observation corresponds to the hypothesis that PHT1;5 is involved in the remobilization of phosphate from older to younger leaves, especially during conditions of Pi-depletion. Himelblau and Amasino demonstrated in 2001 that approximately 78% of stored Pi is transported from older leaves, regulating the Pi homeostasis in the plant during conditions of limitation.

Multiple alignments of protein sequences were conducted in ClustalW and it was discovered that a large proportion of the sequences of high-affinity transporters between different species are conserved. This indicates that structural and functional properties of the Pi/H^+ transporters have been conserved, emphasising its evolutionary importance through the different species.

CHAPTER 4

Expression and functional analyses of *PHT*1;5 in *Escherichia coli* mutant strains

4.1 Introduction

Since as early as the 1940's scientists like Monod have been researching the phosphate transport systems that *E. coli* harbours. Over the years these uptake systems have been investigated and fairly well defined. Basically, inorganic phosphate is transported through either a low or high affinity system, depending on the external conditions, which have been designated respectively as the Pit or Pst transport systems. The properties of these systems have been studied by Rosenberg *et al.* (1977) in mutant *E. coli* strains where only one system was active at a time. Since then these systems have been characterised in more depth and successfully used as heterologous expression systems where similar plant genes have been characterised (Kuhlbrandt and Wang, 1991; Kim *et al*., 1998; Uozomi *et al*., 1998; Pavon *et al*., 2008).

E.coli utilises the low affinity system, Pit, to transport inorganic phosphate (Pi). This system is constitutively active and only functional when sufficient Pi, more than 20 µM, is available in the surrounding area (Rao and Torriani, 1990). This low affinity system permits the movement of Pi by means of proton-motive force and is therefore proposed to be a single-membrane-component transport system. The fact that this system is energized by a proton-motive force disables phosphate transport in the presence of respiration inhibitors and uncouplers.

During times of Pi limitation an enzyme, alkaline phosphatase, hydrolyses organic phosphates to Pi and another system is activated for Pi uptake. The high affinity system, Pst (SCAB), is a phosphate specific transport system which is activated when external phosphate conditions are lower than 20 µM (Rao and Torriani, 1990). This system acts as an operon and involves the activation of several genes, *pst*S, *pst*C, *pst*A and *pst*B, which are all necessary for

the successful transport of phosphate inside the *E.coli* cell (Figure 7). The Pst system is dependent upon the availability of a Pi binding protein, PiBP, in order for it to be functionally active. This PiBP, coded for by the *pst*S gene (Figure 12: A), is extremely specific for Pi with a Km of approximately 1 µM. The two hydrophobic genes from this system, *pst*A and *pst*C, are integral membrane proteins and is believed to structure a membrane pore through which Pi is channelled (Figure 12: B). The final gene involved in this cascade is the *pst*B gene which is bound to *pst*A. This gene is believed to function as an energy coupler (Cox *et al*., 1988). ATP activates *pst*B causing the Pi to be released from the PiBP (Figure 12: B) and bound by the membrane pore. The energy that is discharged from this process enables the transported Pi to become unbound (Figure 12: C) and made available for use by the bacterium. Evidently the Pst system transports Pi at the detriment of ATP (Hoffer *et al*., 2001).

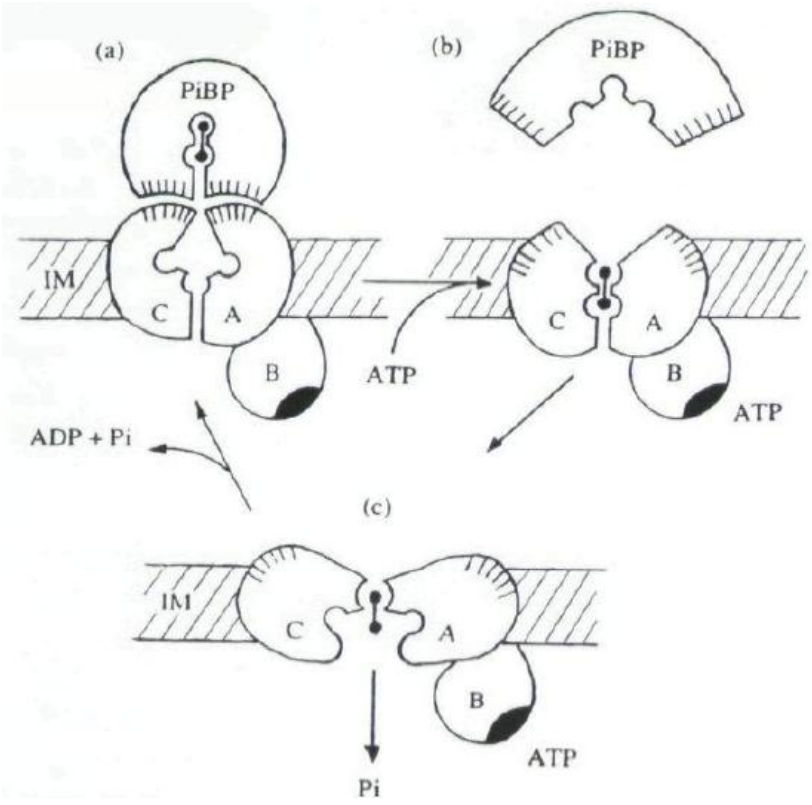

Figure 12: Illustration for Pi transport via the Pst system in *E.coli*. Model drawn by Rao and Torriani, 1990

4.2 Methodology

4.2.1 Materials and *Escherichia coli* strains

All chemicals used during this study were obtained from either MERCK® (Wadeville, Gauteng) or from SIGMA® (Steinheim, Germany), unless otherwise specified. All primers were from Whitehead Scientific (Pty) Ltd. Restriction enzymes used during this study were from Fermentas® (Inqaba, South Africa). All glassware was washed with concentrated HCL to remove traces of phosphate contamination. Media was made sterile in an autoclave for 20 minutes at a temperature of 121^{o} and pressure of 103 kPa.

Commonly used *Escherichia coli* strain, DH5α, was used for all cloning purposes and initial phosphate uptake experiments. *E.coli* mutants (*pst*A, *pst*B, *pst*C, *pst*S), containing deleted genes on the high affinity phosphate uptake *pst* operon, were obtained from the *E.coli* Genetic Stock Centre (http://cgsc.biology.yale.edu/). The *E.coli* mutant strain, CE1491, was a kind gift from Dr J Tommassen at the Department of Molecular Biology and Institute for biomembranes, Utrecht University, The Netherlands. The BL21 Codon Plus RIPL (Stratagene®) *E.coli* strain was used for protein expression.

4.2.2 Isolation of *PHT*1;5

Wild type, Col-0 ecotype, *Arabidopsis thaliana* plants were phosphate stressed in order to express the gene of interest. These experiments were previously conducted and described by Zwiegelaar (2010) where he grew plants on palm peat, watering it with MS salts that contained either 0 mg L^{-1} or 400 mg L^{-1} KH_2PO_4. These phosphate stress treatments were conducted for a period of two weeks until plants started to flower.

RNA Isolation

RNA extractions of phosphate stressed plant material were done according to the method described by White *et al.* (2008). In short, 200 mg of leaf tissue was homogenized in liquid nitrogen with the use of a mortar and pestle. The grounded tissue was supplied with 1.2 ml preheated (65^{o}C) CTAB RNA extraction buffer (2% CTAB, 2% PVP, 100 mM Tris-HCL [pH 8.0], 25 mM EDTA, 2 M NaCl and 0.5 g L^{-1} spermidine) with additionally added 3% β-mercaptoethanol. The mixture was vortexed in order to ensure equal distribution and placed at 65^{o}C for 30 minutes followed by centrifugation (Biofuge, Heraues) for 10 minutes at 13000 x *g*. The supernatant was recovered and an equal volume of chloroform/isopropanol (24:1) was added to the tube. The suspension was vortexed and centrifuged for 15 minutes at 13000 x *g* at 4^{o}C in a desktop microfuge (Biofuge, Heraues). This extraction step was repeated twice. LiCl (final concentration 2M) was added to the supernatant and incubated at 4^{o}C overnight to precipitate the RNA. This sample was then centrifuged for 60 minutes at 13000 x *g*, at 4^{o}C. The pellet was washed with 70% ethanol and resuspended in 30 µl sterile water. A gDNA removal was conducted on the RNA with DNaseI (Promega®) treatment according to the manufacturer's instructions. All RNA samples were stored at -80^{o}C.

Gene amplification

First strand cDNA was synthesised from RNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas®) utilising the Oligo (dT)18 primers according to manufacturer's instructions. RT-PCR was carried out with KAPA HiFi™ DNA Polymerase using the following set of primers, fw: 5'-CAAGATTTTCTCTAGAGTGACTGAACAAC-3' and rev: 5'-GAGTAACACAAAATAATTCTAGAGGGACTTTTCTACCGG-3' designed to amplify the *PHT*1;5 gene. These primers had an added *Xba*I restriction sites for simplified ligation procedures (Zwiegelaar, 2010). All PCR reaction mixtures contained 10 µM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 0.5 units of polymerase and approximately 100ng DNA. The PCR setup were as follow, an initial denaturation step at 95°C for 2 minutes followed by 35 amplification cycles (denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds and an extension at 72°C for 1 minute), a final extension at 72°C for 2 minutes.

All RNA procedures were done in conjunction with the housekeeping gene, actin, as positive control. This entailed the use of actin specific primers: fw: 5'– TCACACTTTCTACAATGAGCT -

3' and rev: 5'- GATATCCACATCACACTTCAT -3'. PCR setup was similar to the setup described above.

Separation of DNA fragments by gel electrophoresis

All DNA/RNA isolations throughout the study have been inspected on a 1% agarose gel, stained with 10 mg ml^{-1} ethidium bromide and visualized under UV light. Agarose gel experiments were conducted in 0.5X TBE buffer at a voltage of between 100 – 120V for at least an hour or until bands have separated sufficiently. TBE buffer (5X) contains 54 g L^{-1} Tris-base, 27.5 g L^{-1} boric acid and 20 ml L^{-1} 0.5 M EDTA (pH 8.0). Band sizes were determined with the help of a lambda *pst* ladder. DNA concentrations were determined on a NanoDrop 1000 (Thermo Fisher Scientific Inc, USA).

Gene cloning

The amplified PCR fragment of *PHT1*;5 was purified with the use of a PCR purification kit (Fermentas®) and ligated into the *Xba*I site of the expression vector pBlueScript SK^- (Figure 13).

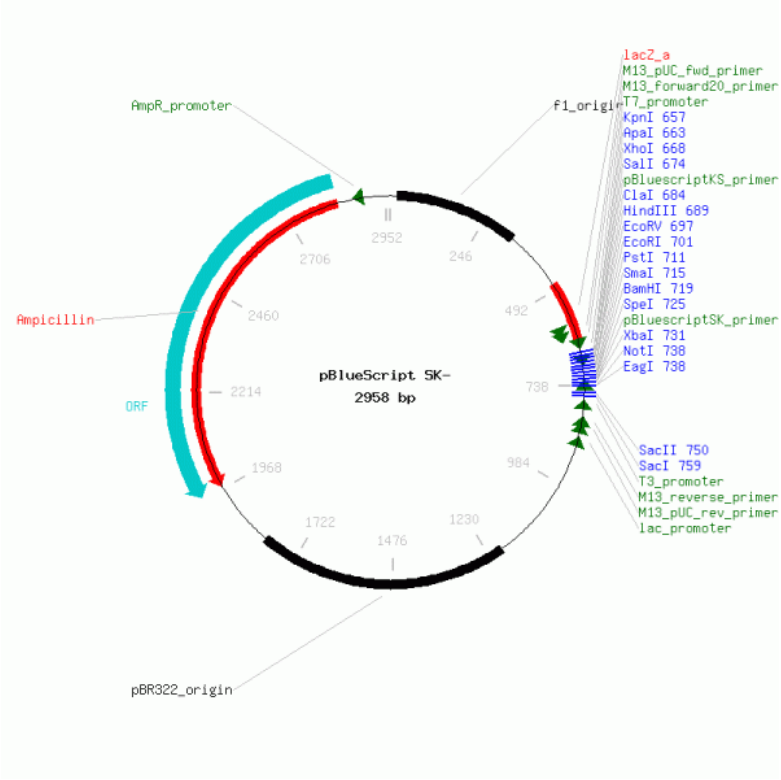

Figure 13: A vector map of pBlueScript (https://www.lablife.org/ct)

The ligation procedure was carried out with T4 DNA Ligase (Fermentas®), according to manufacturer's instruction, for a period of 12 hours at 4°C. The ligation was subsequently followed by transformation of the competent *E.coli* strains.

4.2.3 Competent cells

Heat shock competent cells were made according to a standard $CaCl_2$ protocol (Li *et al*., 2010). In short, a single colony was grown in liquid Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast, 10g L^{-1} NaCl) (Bertani, 1951), supplemented with the necessary antibiotics. Overnight cultures were grown at 37°C, shaking at 200 rpm till an OD_{600} of 0.6. The culture was incubated on ice for 15 minutes and centrifuged at 4000 x *g* for 10 minutes.

The pellets were resuspended in 15 ml cold 0.1 M $CaCl_2$, incubated on ice for 15 minutes and centrifuged as before. The final pellet was resuspended in 4 ml cold 0.1 M $CaCl_2$ and incubated on ice for a further four hours before aliquots of 100 µl was frozen at -80^{o}C.

Electro-competent cells were made according to the manufacturer of the Bio-Rad GenePulser Xcell™ electroporator. This method is similar to that of the heat shock, except that 10% (v/v) glycerol is used in consecutive steps which include the addition of 500 ml, 250 ml, 20 ml and 2 ml glycerol respectively. In between each step the cultures were centrifuged at 4000 x *g* for 15 minutes at 4^{o}C.

4.2.4 Transformation of *E.coli* strains

Electrocompetent DH5α *E.coli* cells were transformed with both empty pBlueScript vector and vector construct, containing the gene of interest, via electroporation with a Bio-Rad GenePulser Xcell™ electroporator according to manufacturer's instructions. Pre-programmed parameters were used for the transformation of 20 µl cells with 1µl plasmid DNA in an electroporation cuvette with an aperture of 0.1 cm. Following the electroporation procedure, cells were incubated for an hour at 37°C in 1 ml SOB (20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.6 g L^{-1} NaCl, 0.5 g L^{-1} KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) with catabolite repression (SOC) medium, shaking continuously at 200 rpm. SOC medium contains filter-sterilized glucose at a final concentration of 20 mM, but is otherwise similar to SOB media as described by Hanahan (1983).

Following the incubation step, 80 µl of cells was spread onto LB medium. The LB medium were solidified with bacteriological agar (Biolab®, Wadeville, Gauteng) and contained 50 µg ml^{-1} ampicillin (Roche, Mannheim, Germany) for selection of positive transformants. Plates were incubated at 37°C for approximately 16 hours and followed by a colony PCR to verify positives clones. The PCR was carried out with T3, 5'-ATTAACCCTCACTAAAGGGA-3', and T7, 5'-TAATACGACTCACTATAGGG-3', primers for pBlueScript, using BIOTAQ™ DNA Polymerase

(Bioline®). Amplification cycles were set according to manufacturer's instructions with a specific annealing temperature of 55°C. Prior to plasmid isolation, 250 ml of LB liquid medium, supplemented with necessary antibiotics, were inoculated with a positive clone and shaken overnight at 200 rpm at 37°C.

E.coli mutants and strain CE 1491 were transformed via a heat shock method. 50 µl of competent cells were thawed on ice followed by the addition of 50ng DNA (empty vector and transformed vector) and incubated for 20 minutes on ice. Subsequently the cells were shocked at a temperature of 42°C for 45 seconds and placed on ice for a further 2 minutes. The cells were rescued with 1ml of SOC and followed by similar incubation and analysis procedures as described for the electroporation transformation.

4.2.5 Plasmid isolation and construct verification

An alkaline lysis method was used to isolated pBluescript-*PHT*1;5 vector DNA according to the method of Untergasser (2006) with minor changes. The volume of the overnight culture was 250 ml LB, and 1 µl of RNaseA (10 mg ml $^{-1}$)(Fermentas®) solution was added after the pellet was resuspended in TE buffer to remove any RNA contamination. This protocol can be viewed for details on the following website: http://untergasser.com/lab/protocols/maxiprep_alkaline_lysis_v1_0.htm.

The pBluescript-*PHT*1;5 construct and the control, pBlueScript empty vector, was digested with *Bam*HI to verify the presence of the *PHT*1;5 gene. The digests were set up, according to Fermentas™ suggestions, as follow: 1U enzyme, 500 ng DNA, 5 µl buffer and sterile water to a final volume of 50 µl. Digests were conducted for two hours at 37°C. Authenticity of the construct was verified by sequencing (Central Analytical Facility, Stellenbosch University). NCBI BLAST analysis was performed to against the Arabidopsis genome to verify the sequence of the cloned gene.

4.2.6 Inorganic phosphate uptake assay

WT *E.coli*, transformed with either the empty vector or gene-containing vector, was grown in 20 ml LB supplemented with 50 µg ml^{-1} ampicillin to an OD_{600} of 0.6. The cultures were centrifuged, as 1 ml aliquots, for 30 seconds at 13000 x *g*. The pellets were washed twice with sterile water and finally resuspended in a K_2HPO_4-KH_2PO_4 buffer (1 M each) to desired concentrations (100 µM, 50µM, 1µM, 500nM and 100 nM), respectively. Aliquots, 100 µl, of these culture suspensions were collected at different short time intervals (2 minutes, 4minutes, 6minutes and 8minutes) and combined with 100µl of a staining solution (1% (w/v) ammonium molybdate and 5% (w/v) $FeSO_4.7H_2O$, solubilised in 0.5 M H_2SO_4). Analysis was done in a microtiter plate using the mQuant spectrophotometer (Biotek Instruments Inc, USA) by measuring the absorbance value at A_{595}.

4.2.7 *E.coli* growth optimization and starvation kill curves

In order to determine the activation point of the high affinity system, starvation kill curves were conducted. *E.coli* cultures were grown overnight in liquid LB media supplemented with necessary antibiotics to an OD_{600} of 0.6. These cultures were centrifuged in a Sorvall® RC5C PLUS centrifuge at 5000 x *g* for 3 minutes in order to pellet the cells. The pellet was resuspended to an OD of 0.1 in modified M9 minimal media (Table 3) containing no phosphate, supplemented with 50 µg ml^{-1} ampicillin, IPTG (Invitrogen®) to a final concentration of 100 µM and Tris-HCl buffer at pH of 7.5. These cultures were then incubated at 37°C with continuous shaking at 200 rpm. A 5 µl aliquot was taken at four different time intervals, 0 hours, 6 hours, 24 hours and 30 hours, which were subsequently spotted onto M9 media plates that contained potassium phosphate buffer at various concentrations which included 0.5, 1, 10, 20 and 50 µM, respectively. These experiments were also utilised to determine the optimal phosphate concentration where expression of the gene is achieved. All subsequent experiments were conducted in a Tris-HCl buffer at pH 7.5 to keep the external conditions constant.

Table 3: Components of M9 salts. These salts are dissolved in distilled water, sterilised and 200 ml added to the M9 media containing 2 ml L^{-1} $MgSO_4$ (1M), 20 ml L^{-1} glucose (20%), 100 µl L^{-1} $CaCl_2$ (1M) and solidified with 15g L^{-1} agarose when necessary

M9 salts	Amount of M9 salts in gram per litre	
	Regular	Modified
Na_2HPO_4-7H_2O	64	/
KH_2PO_4	15	/
NaCl	2.5	10
NH_4Cl	5	5
KCl	/	10

4.2.8 *E.coli* growth curves

In order to determine growth rates of *E.coli* cultures containing either the empty or transformed vector, growth curves were done on all strains. In order to avoid biased results, the experiments included three biological (three different cultures) and three technical (three independent OD readings of each culture) replications. Starter cultures were grown overnight, as previously described, in 2 ml LB medium supplemented with the necessary antibiotics. These cultures were centrifuged in a Sorvall® RC5C PLUS centrifuge at 5000 x *g* for 5 minutes and the pellet resuspended 10 ml M9 media, without the addition of phosphate, for starvation. Following starvation, the cultures were diluted to an OD_{600} 0.1 in 50 ml M9 media containing the appropriate phosphate concentration and supplemented with IPTG. The OD was measured on a µQuant spectrophotometer (Biotek Instruments, Inc) every 2 hours.

4.2.9 Protein analysis

Gene cloning

The pProEX™ HT prokaryotic expression system (Life Technologies™) was used for analysis of the PHT1;5 protein *in vivo*. The expression vector of choice was pProEX HTc and the *PHT*1;5

gene was cloned into the *Not*I site. Cloning of the gene entailed amplification with gene specific primers (see section 3.2.3 for details of gene amplification) and ligation into the pGemT®-Easy vector (Promega™) according to manufacturer's protocol. *PHT*1;5 was digested out of pGemT®-Easy as a *Not*I fragment and sub-cloned in-frame into the *Not*I site of pProEX HTc. The vector construct was transformed into the BL21 CodonPlus® RIPL *E.coli* strain (Stratagene™) (*E. coli* B F– *ompT hsdS*(rB– mB–) *dcm*+ Tetr *gal* λ(DE3) *endA* Hte [*argU proL* Camr] [*argU ileY leuW* Strep/Specr]) via heat shock, as previously described, and selected for on LB media supplemented with 50 µg ml^{-1} ampicillin, 34 µg ml^{-1} chloramphenicol and 10 µg ml^{-1} streptomycin. The presence of *PHT*1;5 in pProEx Htc was verified with colony PCR using the following primers, fw: 5'-AGCGGATAACAATTTCACACA-3', rev: 5'-GAGTAACACAAAATAAT TCTAGAGGGACTTTTCTACCGG-3'. PCR reaction mixtures contained 10 µM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 0.5 units of polymerase and approximately 100ng DNA. The PCR setup were as follow, an initial denaturation step at 95°C for 2 minutes followed by 35 amplification cycles (denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds and an extension at 72°C for 1 minute), and a final extension at 72°C for 2 minutes. DNA sequencing was conducted at the Central Analytical Facility, Stellenbosch University, to verify construct authenticity.

Protein extraction from *E.coli* cultures

A single colony of the BL21 strain, harbouring either the empty vector or a vector containing *PHT*1:5, was grown overnight in 5 ml LB medium supplemented with the necessary antibiotics. These cultures were subsequently inoculated into 250 ml LB media and incubated at 37°C with shaking at 200 rpm, until it reached an OD_{590} 0.6. Once the required OD_{590} was reached, IPTG was added to final concentration of 1 mM to start the induction process. Subsequently, 1 ml aliquots of the cultures were taken at 6 different time points: (0) - just after the addition of IPTG, (1) – one hour, (2) – two hours, (3) – three hours, (4) – four hours and (5) – 18 hours. The aliquots were frozen at -80°C. During each interval the OD_{590} was determined. All absorbance readings were done in a Pharmacia LKB Utrospec III® spectrophotometer.

Protein extractions entailed the thawing of the cell aliquots for 15 minutes on ice. The aliquots were subsequently centrifuged in desktop microfuge for 2 minutes, 13 000 x *g* at 4°C. The pellets were resuspended in 200 µl of a 2x SDS loading buffer (0.09 M Tris-HCL [pH

6.8], 20% [v/v] glycerol, 2% [w/v] SDS, 0.02% [w/v] bromophenol blue and 0.25 M DTT). Lysis was achieved by boiling the resuspended pellets at 95°C for 5 minutes, followed by a centrifugation step of 10 minutes, 13 000 x *g* at room temperature. The supernatant of the samples were analysed with SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE

The SDS-PAGE gel consisted of two layers. First, the 8% resolving gel containing 2.3 ml water, 1.3 ml acrylamide (30%), 1.3 ml Tris (1.5 M, pH8.8), 0.05 ml SDS (10%), 0.05 ammonium persulfate (10%) and 0.003 ml TEMED. Secondly, the stacking gel contained 1.4 ml water, 0.33 ml acrylamide (30%), 0.25 Tris (1 M, pH 6.8), 0.02 ml SDS (10%), 0.02 ml ammonium persulfate (10%) and 0.002 ml TEMED. Electrophoresis was conducted in a 1X SDS buffer made from a 5X stock solution (72 g L^{-1} glycine, 15g L^{-1} Tris and 5 g L^{-1} SDS) for approximately two hours at a current of 140V.

Following electrophoresis, the gel was washed three times with milliQ water for intervals of 10 minutes each. The SDS gel was then incubated in fixing solution (25% isopropanol and 10% acetic acid) for 15 minutes and washed three times as before. Staining of the gel was done with a PageBlue™ Protein Staining Solution (Fermentas®) for at least 12 hours where after it was washed with water until protein bands were visible. All incubation steps were performed on a Heidolph UNIMAX 1010 shaker, shaking at 200 rpm. The marker used to determine protein band sizes was the PageRuler™ prestained protein ladder (Fermentas®).

4.3 Results and Discussion

4.3.1 Expression conditions and isolation of the *PHT*1;5 gene

A recent study done in 2010 by Zwiegelaar aimed at investigating the expression pattern of the *PHT*1;5 gene during phosphate rich and depleted conditions. Zwiegelaar utilised *Arabidopsis thaliana* WT and *PHT*1;5 T-DNA insertion mutant lines (obtained from NASC European *Arabidopsis* stock centre) and indicated through Northern blot analysis that the gene was highly expressed in leaf tissue when the external phosphate concentration was lower than 50 mg L^{-1}. Zwiegelaar harvested leaf material from the WT phosphate-stressed plants and kindly donated it to this study for further analysis. RNA was successfully extracted

from this tissue (Figure 14: A) and RT-PCR was subsequently performed with gene specific primers designed to amplify out the 1.7 kb cDNA corresponding to the full coding region of the *PHT*1;5 gene (Figure 14: B). These primers were designed to contain internal *Xba*I sites for simplified down-stream cloning procedures and evidently *PHT*1;5 cDNA was cloned in frame into the *Xba*I site of the bacterial expression vector pBlueScript SK$^-$ (Invitrogen®) and the gene was successfully propagated in an *Escherichia coli* based system.

In order to confirm the presence of *PHT*1;5 a restriction digest was performed with the enzyme *Bam*HI on either the empty vector or the vector construct that contained the gene of interest. This enzyme digests only pBlueScript once and resulted in approximate fragment sizes of 2985 bp and 4685 bp respectively for the vector and the gene-containing vector construct. The results obtained from the digest were analysed on an agarose gel (Figure 14: C) and is concurrent with the given band sizes that should have been obtained. Sequence analysis confirmed the correct frame and orientation of *PHT*1;5 inside the vector construct. The authenticity of the gene sequence was confirmed by a BLAST analysis against the *Arabidopsis* genome and no mutations were detected. Effective gene propagation was achieved and transformation of electrocompetent *E. coli* cells with the construct (pBlueScript-*PHT*1;5) and empty vector (pBlueScript) was successfully conducted.

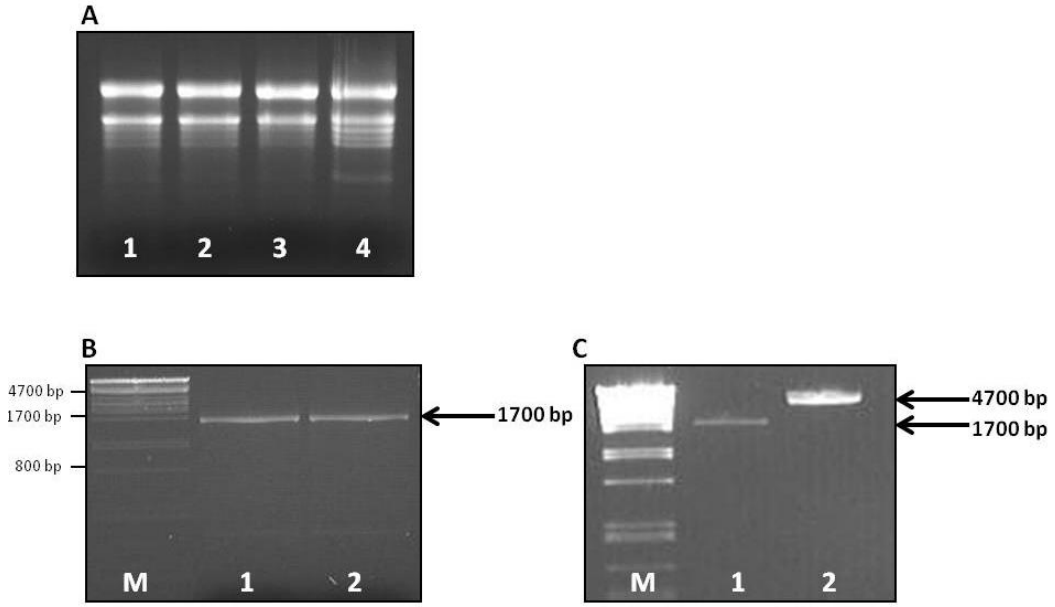

Figure 14: M = molecular marker Lambda *pst*I (**A**) Lanes, 1-4 – Isolated RNA from phosphate stressed WT *Arabidopsis thaliana*, (**B**) Lanes, 1 and 2 – RT-PCR amplification of *PHT*1;5, (**C**) Lanes, 1 – *PHT*1;5 fragment, 2 – pBlueScript-*PHT*1;5 construct

4.3.2 Inorganic phosphate uptake assay in transformed WT *E. Coli*

Phosphate uptake assays were performed in an attempt to investigate the ability of the *PHT*1;5 gene to increase phosphate transport in a bacterial system. This inorganic P uptake assay was only utilised during the first trial of *E.coli* experiments and was only conducted with the wild type strain, DH5α. The reason for the decision was the hypothesis that the gene, being constitutively expressed in the pBlueScript vector, would increase the rate of phosphate uptake regardless of the strain or medium used.

The assays were conducted in LB medium which was supplemented with a range of phosphate concentrations in order to determine whether there was a concentration preference displayed by the *PHT*1;5 gene. This range included concentrations from 100 µM, 50µM, 1µM, 500nM to as low as 100 nM and covers phosphate the range in which high-affinity transporters are usually activated. The total phosphate content per milligram of protein was determined for cultures in order to avoid conflicting measurements.

Inconsistent results (data not shown) were continuously obtained but mostly indicated one of two scenarios, either that there was no change between the control and transformed phosphate contents or that the phosphate contents in the transformed culture decreased over time. This latter observation resulted in a new hypothesis which suggested that the *PHT*1;5 gene might encode for a transporter involved in the export of phosphate rather than import or that it might function as a uniporter, but this assumption was just speculation and would need further analysis.

Standard assays were conducted with only the LB medium in order to determine what the degree of phosphate contamination might be. The assay indicated that LB medium is already saturated with phosphate, containing approximately 3mM, interfering with the results and saturating the bacteria with internal phosphate concentrations, possibly the cause of cells exporting phosphate rather than import.

The subsequent assays were conducted in a similar way with the exception that the cultures were subject to a period of 30 minutes incubation in only water in order to ensure that they utilise all their internal phosphate. When the stressed cultures were introduced to the various phosphate concentrations, similar inconsistent results were obtained and it was decided that the assay was not sensitive enough to detect minor changes in the bacterial phosphate uptake mechanisms. The phosphate uptake assay failed to yield consistent results and it was decided to abort the experiment all together and continue with complementation studies. The complementation studies included the use of *E.coli* mutant strains which would possibly clarify biased conclusions obtained during the phosphate uptake assay.

4.3.3 Growth optimisation and starvation kill curves of *E.coli* cultures

The various mutant strains used during the complementation studies were from the Keio collection of single-gene knockouts and all displayed a mutation in one of the genes necessary for the Pst high affinity phosphate uptake system, respectively. *E. coli* cultures were grown normally in LB media containing approximately 3mM phosphate as determined by means of the phosphate uptake assay previously described. This suggested that the

bacteria is already saturated with phosphate internally and would therefore not express any high affinity uptake mechanisms until phosphate was severely limited. A second challenge that appeared was the fact that the bacteria started to die off in a short period of time. This phenomenon was investigated by examining the external pH conditions which could be decreased as a cause of H^+ ions being exported. This would hold true due to the fact that it is proposed that the PHT1;5 transporter mediates the movement of phosphate in exchange for H^+-ions. The pH of the media was severely affected as soon as growth of the cultures was initiated (Figure 15: A). External pH conditions were therefore kept constant by supplementing the media with Tris-buffer at a pH of 7.5 (Figure 15: B).

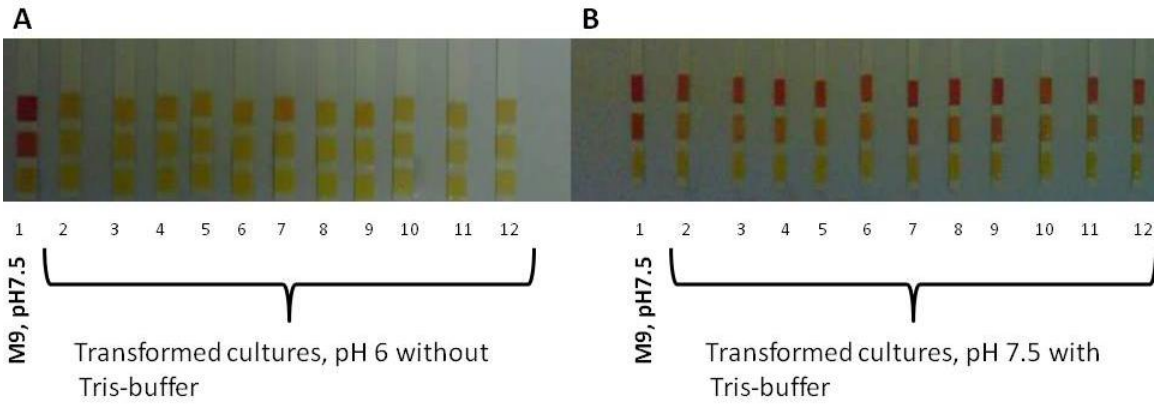

Figure 15: pH determined by use of Macherey-Nagel 100 colour-fixed indicator sticks (pH4.5-10). (**A**) pH values before Tris buffer; Lanes, 1 – M9 media, 2 – WT untransformed, 3 – WT pBlueScript, 4 – WT *PHT*1;5, 5 – pstA pBlueScript, 6 – pstA *PHT*1;5, 7 – pstB pBlueScript, 8 – pstB *PHT*1;5, 9 – pstC pBlueScript, 10 – pstC *PHT*1;5, 11– pstS pBlueScript, 12 – pstS *PHT*1;5 (**B**) pH values after tris buffer; Lanes, 1 – M9 media, 2 – WT untransformed, 3 – WT pBlueScript, 4 – WT *PHT*1;5, 5 – pstA pBlueScript, 6 – pstA *PHT*1;5, 7 – pstB pBlueScript, 8 – pstB *PHT*1;5, 9 – pstC pBlueScript, 10 – pstC *PHT*1;5, 11– pstS pBlueScript, 12 – pstS *PHT*1;5

Positive *E. coli* transformants for the mutants *pst*A, *pst*B, *pst*C and *pst*S (*PHT*1;5-pBlueScript construct and control, empty pBlueScript) were confirmed by means of colony PCR. Results from the PCR were analysed on a 1% agarose gel and band sizes of 1.7 kb were observed, indicative of successful transformation events.

The transformed (*PHT*1;5) and control (empty pBlueScript) cultures were subject to starvation kill curves on M9 minimal media plates. This experiment was conducted because

cultures were grown in LB media which saturates the internal phosphate conditions of the bacteria. Thus, the cultures were stressed in liquid minimal M9 media, without supplementation of phosphate, for periods of 0, 12, 24 and 36 hours, respectively in order to determine what period of time should pass before the internal phosphate would become depleted and compensated for by the transporter. The solid minimal media, onto which these cultures were spotted after the respective time points, was supplemented with various phosphate concentrations ranging from 0 µM, 0.5 µM, 1 µM, 20 µM, 40 µM and 100 mM to establish an approximate value at which the transporter is optimally activated. It was also necessary to determine the depletion point on minimal phosphate concentrations to be able to prove that a complementation event could occur.

The starvation kill curve plate research was performed for three reasons; firstly to establish if there is a time point at which internal phosphate would deplete, secondly to approximate at what concentration the transporter would optimally be activated and thirdly to determine which of the mutant *E.coli* strains could possibly be used for complementation experiments. It was observed (Figure 16: B) that the optimal time of depletion was achieved for one of the *E.coli* mutant strains, *pst*S, and that after 24 hours of growth without phosphate was enough for the strain to possibly start utilising the high-affinity PHT1;5 transporter at a concentration of 20 µM. The same cultures were spotted onto minimal media which was supplied with sufficient amounts of phosphate in order to indicate that cell death was a rare possibility (Figure 16: A).

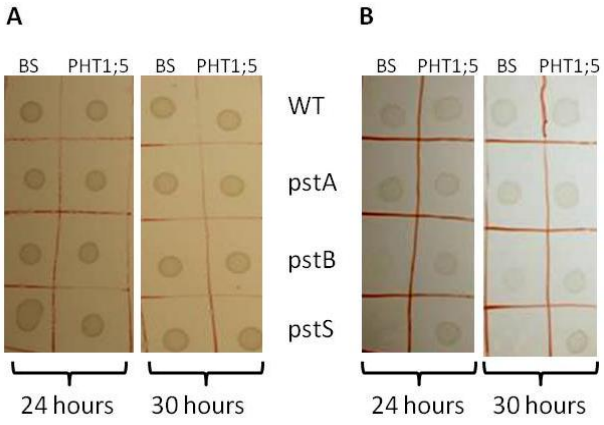

Figure 16: Images of a starvation kill curve plates on M9 minimal media. BS = cultures harbouring an empty pBlueScript control, PHT1;5 = cultures harbouring the pBlueScript-*PHT*1;5 construct, WT = wild type *E. coli*, mutant strains = pstA, pstB and pstS (strain pstC failed to grow) (**A**) Cultures grown on media containing 100 mM phosphate to indicate that cell death did not occur during incubation periods of 24 and 30 hours, (**B**) Cultures grown on media containing 20 µM phosphate, indicating that after 24 hours the *pst*S *E. coli* mutant strain is a possible candidate for complementation studies.

The initial train of thought before the study started was that the *pst*S mutant was the most likely strain to be complemented with the PHT1;5 transporter. The *pst*S gene encodes for a phosphate binding protein and we hypothesised that the bacteria would not be able to transport phosphate without this binding ability, unless it is supplied with another system which would be able to compensate for its disability. The other system evidently included the PHT1;5 transporter and according to the results observed stated the possibility of complementation. The fact that growth of the transformed (*PHT*1;5) *pst*S strain was visible in the micro-molar concentration range of supplied phosphate initiated the argument that PHT1;5 might function as a high affinity transporter and the transformed *pst*S strain was subject to further growth experiments to test the theory.

4.3.4 Complementation studies on *E. coli*

In the event of establishing the phosphate depletion time for bacterial cultures, mutant strains were tested for complementation by the *PHT*1;5 gene on minimal media. The most promising strain, *pst*S, was chosen and tested for complementation on a variety of phosphate concentrations, although the previous kill curve experiments indicated a

concentration of approximately 20 µM, it was necessary to determine whether a concentration range existed in which this transporter were to be active.

Complementation experiments were conducted on M9 minimal media and indicated the possibility of the PHT1;5 transporter to complement the *pst*S mutant strain on a range of concentrations. As expected, the 20 µM phosphate concentration displayed the most promising results of complementation (Figure 17) which was in conjunction with the Km values for other high affinity transporters (Leggewie *et al*., 1995; Daram *et al*., 1999).

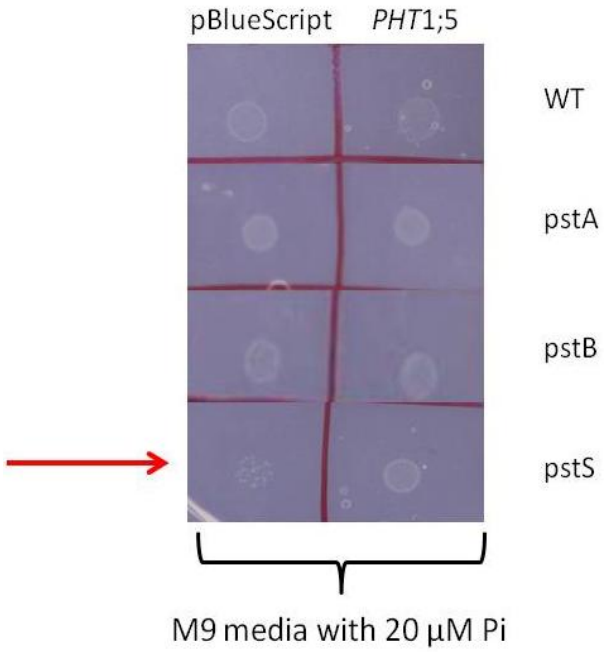

Figure 17: Putative complementation results for *E. coli*. M9 minimal media plate containing 20 µM phosphate, indicating the possible complementation for *pst*S mutant strain. pBlueScript = cultures harbouring an empty pBlueScript vector as control, PHT1;5 = cultures harbouring the pBlueScript-*PHT*1;5 construct, WT = wild type *E. coli,*mutant stains =pstA, pstB, pstS

Once the desired phosphate concentration of 20 µM was established, growth curves were conducted for the transformed and control *pst*S mutant strain. The curves were to indicate that the transformed (*PHT*1;5) display an increased growth rate as opposed to the control, but this was not the case. On the contrary, the growth curves indicated that there was no significant difference in growth rate between the transformed and control cultures (Figure

18) which evoked the question of whether there was another internal system in the bacteria that could compensate for this lack of the pstS phosphate binding protein. Indeed when literature was approached again, it became evident that the inability of the *pst*S mutant strain to transport phosphate can be substituted for by the internal low affinity systems, PitA and PitB, of the bacteria. Hoffer and colleagues demonstrated in 2001 that the activity of wild type PitA or PitB were able to restore Pi regulation in a PstS mutant strain. These results indicated that Pi-regulation mediated by the Pst system could still take place in the absence of the PstS binding protein. This rendered all previous results as unreliable.

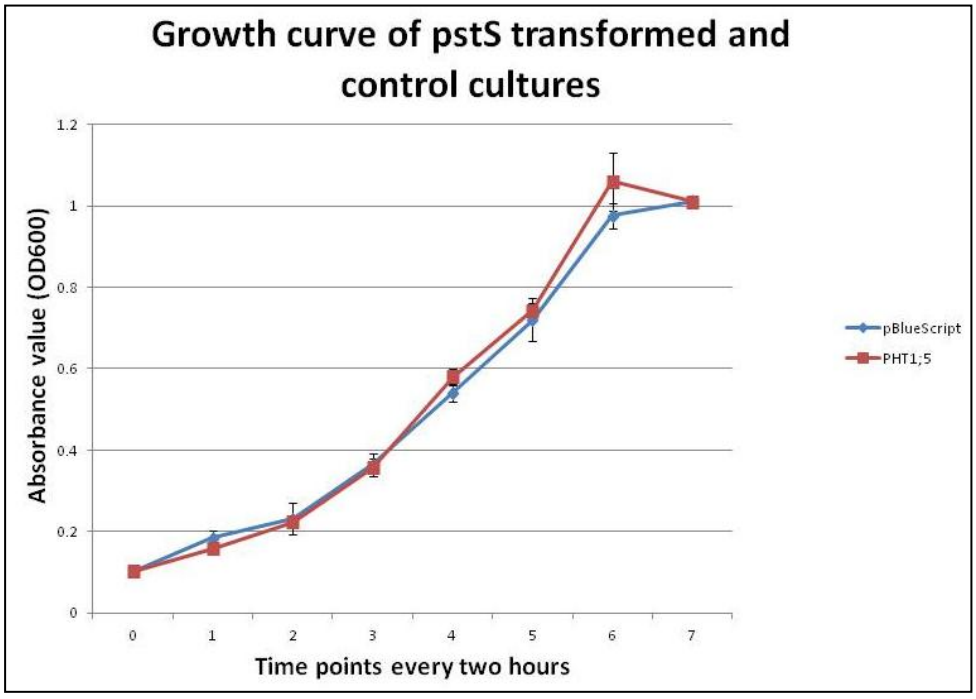

Figure 18: Graph indicating the growth rates of transformed (*PHT*) and control (pBlueScrip) pstS cultures. Data presented in this graph are means derived from three independent experiments with three replicates. Errors of the standard deviation of the means are indicated as bars.

After establishing that the single-gene knockout strains failed to yield consistent and reliable results, it was decided to investigate complementation of a different strain, CE1491, which is absolutely unable to transport inorganic phosphate. This strain harbours no high or low affinity phosphate uptake system and a new complementation journey was attempted. In

order to allow the strain to grow under normal conditions, it was necessary to supplement all media with glycerol-3-phosphate (Hoffer and Tommassen, 2001). Transformed (*PHT*1;5) and control (empty vector) bacterial cultures were successfully propagated and verified via colony PCR as before. The PCR was analysed on a 1% agarose gel and colonies that were transformed with the *PHT*1;5 vector construct indicated band sizes of 1.7 kb which was in concordance with the size of the gene (Figure 19). The PCR analysis of the control colonies, harbouring the empty pBlueScript vector, indicated band sizes of approximately 200 bp which was indicative of successful transformation events (Figure 19).

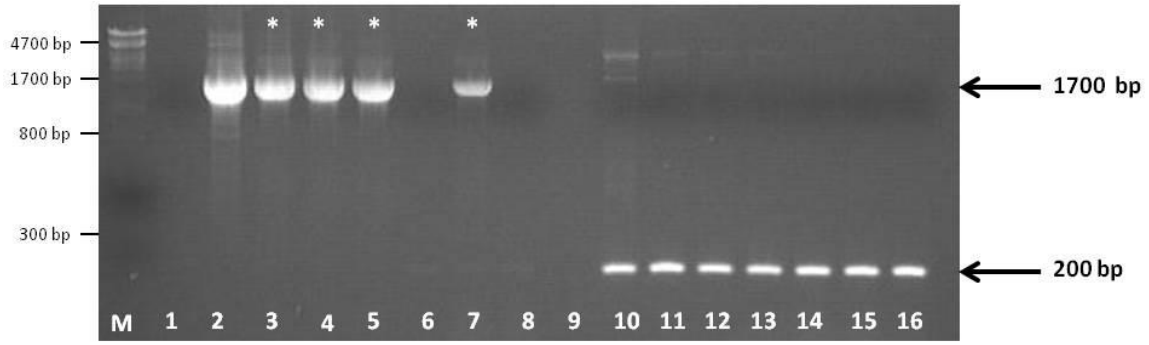

Figure 19: PCR analysis of CE1491 colonies containing either the pBluescript-*PHT*1;5 construct or the empty vector. Lanes, M - Lambda *pst*I molecular marker, 1- Negative water control, 2- Positive control for *PHT*1;5, 3-7 *PHT*1;5 amplified 1700 bp PCR fragments (positive colonies indicated with asterix), 10 - Positive control for empty vector, 11-16 - pBlueScript 200 bp PCR fragments (empty vector)

The experimental design for the CE1491 strain was similar as for the other strains and after starvation periods, cultures were spotted onto minimal media plates with the same phosphate concentrations as described previously. The *PHT*1;5 gene was unable to complement the CE1491 strain on minimal plates, even after the addition of several amino acids and vitamins. It could be a possibility that this strain is unable to function properly in the absence of glycerol-3-phosphate, which must be excluded from the media when complementation studies were to be done. This statement was tested and cells were grown on minimal media supplied with this phosphate and still growth was arrested on minimal

media. It is suggested that growth of this strain on minimal media needs further optimisation with regards to supplements and concentrations. Another argument that had been raised was whether the expression of the foreign gene, *PHT*1;5, might be toxic to this sensitive strain of bacteria. In order to prove the statement, protein expression was done in a different strain, BL21 CodonPlus-RIPL, which was also executed to determine whether functional protein expression of PHT1;5 can be achieved in a bacterial system.

4.3.5 Functional analyses of the PHT1;5 protein in *E. coli* cells

During the investigation of heterologous PHT1;5 protein expression in *E.coli,* it was required that a special strain should be utilised. This BL21 CodonPlus-RIPL strain enables the production of heterologous proteins in the bacterial system which is often challenged by the shortage of certain tRNAs. These tRNAs are required for the high-level expression of proteins in plants and can often impede the translation process when expressed in bacteria. The BL21- RIPL strain is equipped with extra tRNAs that include *argU*, *ileY,* and *leuW* as well as the *proL* and is therefore often able to liberate the production of heterologous proteins from species that have GC- or AT-rich genomes.

The vector of choice for foreign protein expression was pProEx-HTc which has been designed for this purpose of expression analysis in *E. coli*. The *PHT*1;5 gene was cloned into the *Not*I site of the multiple cloning site of the vector and successfully transformed into the BL21 strain. Transformants were verified via colony PCR and subsequently the fragments were analysed on a 1% agarose gel (Figure 20: A). PCR fragments of approximately 1.7 kb were obtained for positive transformants and the DNA was further analysed via restriction digests with HindIII to confirm whether the correct vector was present. Digests were also analysed on a 1% agarose gel (Figure 20: B). *Hind*III only digests the vector once and does not cut inside the gene, therefore fragment sizes of 4.75 kb and 6.45 kb for pProHtc and pProHtc-*PHT*1;5 were expected, respectively. These fragments were observed on the agarose gel (Figure 20: B) and DNA was subject to further sequencing analysis which confirmed the correct frame of the construct.

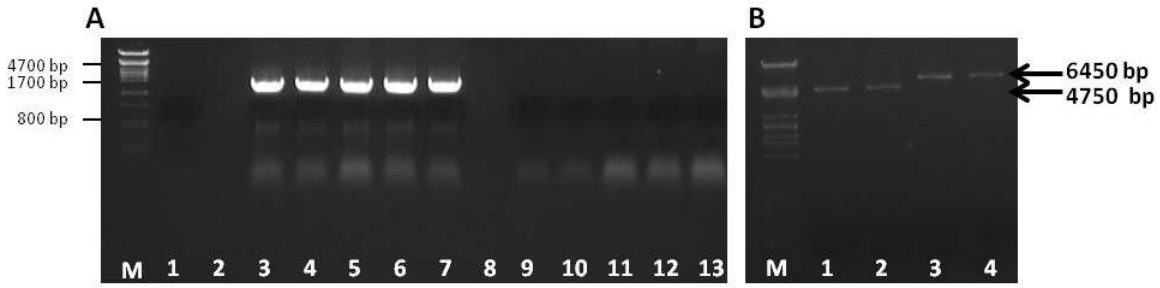

Figure 20: (**A**) PCR analysis of pProHTc transformed (*PHT*1;5) and control (empty vector) constructs; Lanes, M - Lambda *pst*I molecular marker, 1- Negative water control, 3-7 - 1.7 kb positive PCR fragments for *PHT*1;5, 9-13 - pProHtc empty vectors amplified with same primers (**B**) Restriction analysis of pProHtc vectors; Lanes, M - Lambda *pst*I molecular marker, 1 and 2 - pProHtc linearised with *Hind*III displaying fragment sizes of 4.75 kb, 3 and 4 - pProHtc-*PHT*1;5 construct linearized with *Hind*III displaying fragment sizes of 6.45 kb

Upon establishing successful transformation of the BL21 strain with the constructs, protein analysis *in vivo* followed. Transformed (pProHtc-*PHT*1;5) and control (empty pProHtc) cultures were grown in LB medium until the desired OD_{590} of 0.6 was reached. Cultures were induced with IPTG and aliquots were subsequently used for protein extraction and analysis. Following protein extractions of the induced culture aliquots, analysis was done on 8% SDS-PAGE gels. The results visible on the gels (Figure 21) were not as expected for the transformed construct. Protein analysis of the control (Figure 21: A) indicated a gradual increase during the incubation period which was expected, thus no technical mistakes were made during the experimental procedures. Upon analysis of proteins from the transformed cultures (Figure 21: B), SDS-PAGE gels indicated no increase of general bacterial proteins, neither was the expected PHT1;5 protein of 59kDa observed. Various reasons exist as to why the expected protein was not observed and these include the possibility of the protein being degraded by the bacteria even before the extraction procedure was carried out. Another reason might be that the foreign protein does not fold correctly in bacterial cultures and would therefore not be functional, leading to possible toxic effects.

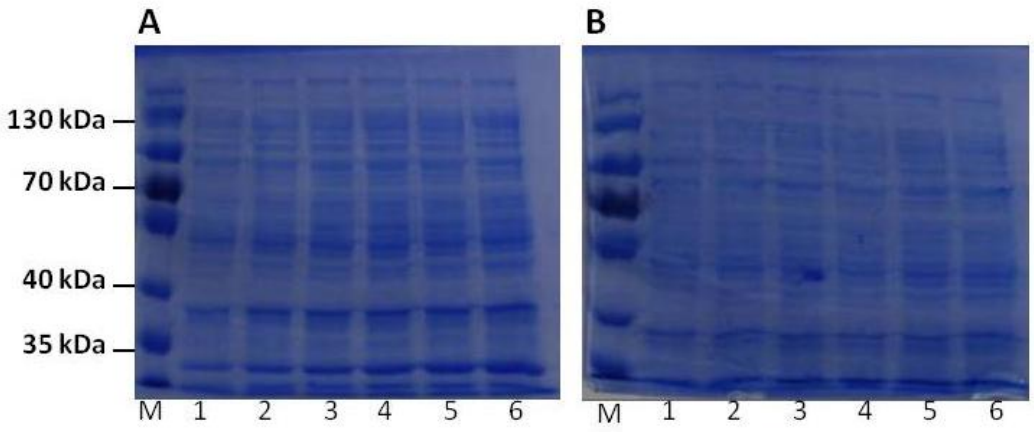

Figure 21: (**A**) SDS-PAGE gel analysis of proteins in the control (empty pBlueScript vector), an increase in proteins over time is visible. Lanes, M- PageRuler™ Pre-stained protein ladder, 1 - Time 0, 2 – one hour, 3 – two hours, 4 – three hours, 5 – four hours, 6 – eighteen hours. (**B**) SDS-PAGE gel analysis of proteins from *PHT*1;5-containing cultures, no increase in any protein visible. Lanes, M- PageRuler™ Pre-stained protein ladder (indicating sizes in kDa), 1 - Time 0, 2 – one hour, 3 – two hours, 4 – three hours, 5 – four hours, 6 – eighteen hours

Probably the main reason for this is that PHT1;5 is a membrane-bound protein which often display toxic characteristics when induced in bacterial cultures. This statement was proven by analysis of growth curve experiments before and after IPTG induction. Growth curves were conducted with three independent cultures to avoid any biological or technical mistakes and the absorbance values were measured from an OD_{590} of 0.1 hourly. During these experiments it clearly demonstrates that growth before IPTG addition is at the same rate for both the transformed and control cultures. As expected by our hypothesis, upon addition of IPTG, transformed cultures display a decrease in growth as opposed to the control (Figure 22), leading to the general impression that the induced PHT1;5 protein display cyto-toxic effects in a bacterial system. To further confirm these statements, cyto-toxicity vs cell death testing could be considered in the future by conducting a colorimetric assay (MTT) which would indicate the survival rate of the bacterial cultures.

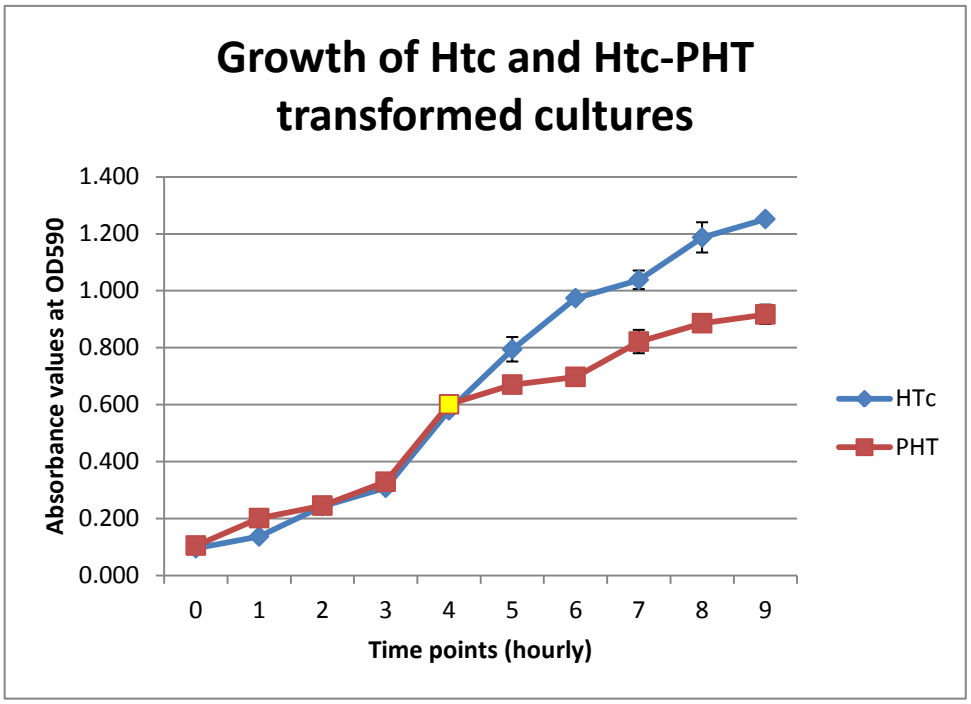

Figure 22: Growth curve of control (empty pProHtc) and transformed (*PHT*1;5-pProHtc construct) BL21 cultures before and after addition of IPTG to test for possible protein toxicity. IPTG was added at time point 4 on the graph (yellow square). Data presented in this graph are means derived from three independent experiments with three replicates. Errors of the standard deviation of the means are indicated as bars.

4.4 Concluding remarks and future prospects

Although *E.coli* is the most extensive system used for heterologous expression due to its rapid growth rate and being a fairly simple system to work with, it is not always successful when analysing some membrane bound proteins (Yin *et al.*2007). Data obtained during this study demonstrates that the bacterial system was unsuccessful and inconclusive regarding the expression and functional studies of the PHT1;5 transporter. These results emphasises the fact that , although *E.coli* is a rapid, simple and high yielding protein expression system, heterologous expression of membrane bound proteins are often challenging for these microorganisms and evoke cytotoxic effects. Another problem that should be considered is that one of the disadvantages displayed by the BL21CodonPlus-RIPL strain is the leaky expression of the T7 polymerase. Although this strain is often used successfully for high-level heterologous protein expression, the leaky T7 often initiates low-level expression of un-induced proteins which might potentially be toxic to the bacterial cultures. During this study

our results are indicative of potentially toxic effects of PHT1;5 to bacterial cultures. It might be useful in future experiments to verify this effect via colorimetric assay (MTT) which would indicate the survival rate of the bacterial cultures.

Because the bacterial expression systems are so easy to work with and cost effective, it would also be useful in future studies to optimize the use of different strains which might be able to overcome toxic effects of membrane-bound proteins. We suspect that PHT1;5 might have toxic properties in bacterial systems and would therefore recommend that future experiments be carried out in BL21-CodonPlus-RIL or BL21-CodonPlus-RP cells. These strains are often recommended to be used during studies of genes that encode for proteins which might potentially be toxic to bacteria. Induction is done by infecting the cells with Lambda CE6 and this method of heterologous protein expression strictly regulates the expression of genes under control of T7 promoter.

Although we failed to yield consistent and confirmative results through the bacterial expression system, we still propose that the PHT1;5 transporter function as a H^+/Pi symporter and can be assumed according to the almost immediate drop in pH conditions after cell growth was initiated.

CHAPTER 5

Expression and functional analyses of *PHT*1;5 in a mutant yeast strain, PAM2

5.1 Introduction

The yeast *Saccharomyces cerevisiae* has been a eukaryotic model system throughout the decades and has developed, like other micro-organisms, numerous strategies to adapt to the nutritional limitations in their environments. Some of these strategies involve the coordinated activation and derepression of various transporter proteins. *S. cerevisiae* have three independent systems involved in the transport and uptake of inorganic phosphate (Pi) (Nieuwenhuis and Borst-Pauwels, 1983). These include one low-affinity and two high–affinity phosphate uptake systems which have been relatively well studied and characterised. The low-affinity system is constitutively active with a high Km value, 770 µM, and is proposed to be optimally active at a pH of 4.5 (Goodman and Rothstein, 1957; Legget, 1961). The derepressible high-affinity systems are encoded for by the *PHO*84 and *PHO*89 genes, respectively, and are only activated during conditions of phosphate depletion. *PHO*84 is an H^+co-transporter with a Km value of 10µM and *PHO*89 is a Na^+co-transporter displaying a Km value around 1µM, respectively optimal at pH ranges of 4.5 and 7.2 (Roomans *et al.*, 1977). These plasma membrane permeases regulate the movement of Pi in and out the cell (Bun-Ya *et al*., 1991; Martinez and Persson, 1998).

The mutant yeast strain, PAM2, is devoid of both these high affinity permeases. However, it harbours a single low affinity transport system in order for it to actively grow during optimal conditions. Being extensively studied, both of these uptake systems in yeast lead to a mostly successful platform for heterologous expression of similar transporters in plants (Leggewie *et al*., 1997; Daram *et al*., 1998; Daram *et al*., 1999; Guo, 2008). The PAM2 strain has gained increasing popularity during studies where plant membrane transporters had to be characterised and was therefore the obvious choice for a eukaryotic expression system during this study.

5.2 Methodology

5.2.1 Materials and yeast strain

All chemicals used during this study were obtained from either MERCK® (Wadeville, Gauteng) or from SIGMA® (Steinheim, Germany), unless otherwise specified. All primers were from Whitehead Scientific (Pty) Ltd. Restriction enzymes used during this study were from Fermentas® (Inqaba, South Africa). Amino acids were from Sigma-Alderich® (Steinheim, Germany) unless otherwise specified. All glassware was washed with concentrated HCL to remove traces of phosphate contamination. Media was made sterile in an autoclave for 20 minutes at a temperature of 121^{o} and pressure of 103 kPa.

The yeast strain utilised for complementation experiments during the study was PAM*2* (Δpho89::*TRP*1; Δpho89::*HIS*3 *ade*2 *leu*2 *his*3 *trp*1 *ura*3) and was a kind gift from Prof. P Daram, Federal Institute of Technology, Zurich, Institute of Plant Sciences. This strain is devoid of its internal high affinity Pi uptake systems, but harbours a low affinity uptake system in order to be operative under normal growth conditions.

The yeast expression vector, pHVX2, was a kind gift from Dr. J Franken from the Institute for Wine Biotechnology, Stellenbosch University, South Africa.

5.2.2 Gene propagation

Amplification of *PHT*1;5

The gene was amplified with KAPA HiFi^{TM} DNA Polymerase using the following set of primers, fw: 5'-CAAGATTTTCTCTAGAGTGACTGAACAAC-3' and rev: 5'-GAGTAACACAAAATAATTCTAGA GGGACTTTTCTACCGG-3'. The PCR reaction mixture contained 10 µM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 0.5 units of polymerase and approximately 100ng DNA. The PCR setup were as follow, an initial denaturation step at 95°C for 2 minutes followed by 35

amplification cycles (denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds and an extension at 72°C for 1 minute), a final extension at 72°C for 2 minutes.

Gene cloning

The amplified *PHT*1;5 gene fragment was phosphorylated with T4 Kinase (Fermentas®) according to manufacturer's instructions. During this procedure approximately 150 ng of DNA was utilised, 2 µl buffer, 1 µl kinase, 2 µl ATP (10 mM) and water to a final volume of 20 µl. Phosphorylation was carried out at 37°C for 20 minutes and the enzyme inactivated at 75°C for 10 minutes. The 2 micron multi copy plasmid (Figure 23), pHVX2, was used as yeast expression vector. The vector (700 ng) was digested with *Xho*I at 37°C for 90 minutes and inactivated at 80°C for 20 minutes. The vector was made blunt-ended with T4 Polymerase (Fermentas®) with the addition of 1 µl dNTP (2 mM) and 0.2 µl T4 polymerase to the inactivated plasmid digest mixture. Blunting was done at room temperature for 5 minutes and the reaction inactivated at 70°C for 10 minutes. The blunted plasmid reaction was cleaned with a PCR clean-up kit from Fermentas® according to manufacturer's instructions. The vector was subsequently dephosphorylated with FastAP alkaline phosphatase (Fermentas®). The dephosphorylation reaction contained 500 ng of plasmid DNA, 2 µl of buffer, 1µl alkaline phosphatase and brought to volume with sterile water. The reaction was incubated at 37°C for 10 minutes and inactivated at 75° for 5 minutes.

The phosphorylated gene fragment and the dephosphorylated plasmid digest were purified with a PCR clean-up kit from Fermentas® and a blunt-end ligation procedure was carried out with T4 ligase (Fermentas®) in a final volume of 20 µl. The ligation reaction contained 300 ng of insert DNA, 100 ng plasmid DNA, 2 µl ligase buffer, 2 µl PEG 4000 (50%) and 1 µl DNA ligase (5U). The ligation reaction was carried out at 22°C for an hour and inactivated at 65°C for 10 minutes.

Electrocompetent DH5α *E.coli* cells were transformed with both empty pHVX2 vector and vector construct, containing the gene of interest, via electroporation with a Bio-Rad GenePulser Xcell™ electroporator according to manufacturer's instructions. Pre-programmed parameters were used for the transformation of 20 µl cells with 1µl plasmid DNA in an electroporation cuvette with an aperture of 0.1 cm. Following the electroporation

procedure, cells were incubated for an hour at 37°C in 1 ml SOB (20 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 0.6 g L^{-1} NaCl, 0.5 g L^{-1} KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$)with catabolite repression (SOC) medium, shaking continuously at 200 rpm. SOC medium contains filter-sterilized glucose at a final concentration of 20 mM, but is otherwise similar to SOB media as described by Hanahan (1983).

Following the incubation step, 80 µl of cells was spread onto LB medium. The LB medium were solidified with bacteriological agar (Biolab®, Wadeville, Gauteng) and contained 50 µg ml^{-1} ampicillin (Roche, Mannheim, Germany) for selection of positive transformants. Plates were incubated at 37°C for approximately 16 hours and followed by a colony PCR to verify positives clones. PCR was carried out with gene specific primers as previously described in this section.

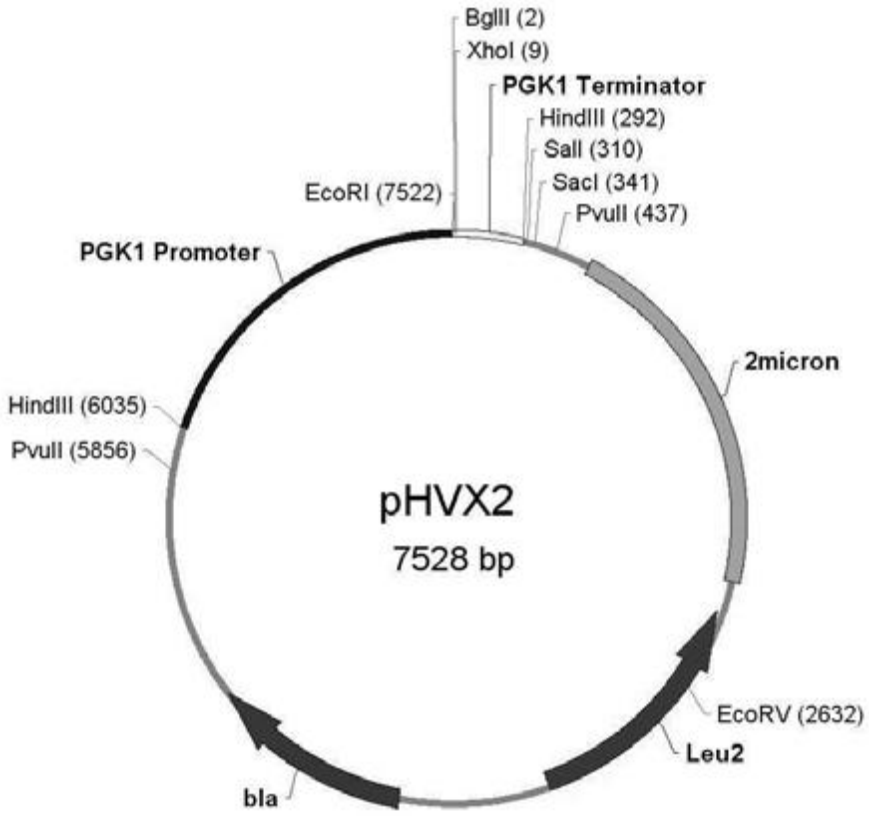

Figure 23: The 2 micron high copy plasmid, pHVX2, for yeast expression.

Plasmid isolation and construct verification

Positive clones, as determined via colony PCR, were selected and subject to various restriction digest analysis. The pHVX2-*PHT*1;5 construct and the control, pHVX2 empty vector, was digested with various enzymes; *Bam*HI, *Bgl*II, *Sac*I, *Kpn*I, *Xho*I, *Sma*I and *Eco*RI respectively or in combination to verify the presence and orientation of the *PHT*1;5 gene. The digests were set up, according to Fermentas™ suggestions, as follow: 1U enzyme, 500 ng DNA, 5 µl buffer and sterile water to a final volume of 50 µl. Digests were conducted for two hours at 37°C.

An alkaline lysis method was used to isolate the positive pHVX2-*PHT*1;5 vector DNA according to the method of Untergasser (2006) with minor changes. The volume of the overnight culture was 250 ml LB, and 1 µl of RNaseA (10 mg ml $^{-1}$) (Fermentas®) was added after the pellet was resuspended in TE buffer to remove any RNA contamination. This protocol can be viewed for details on the following website: http://untergasser.com/lab/protocols/maxiprep_alkaline_lysis_v1_0.htm.

Authenticity of the construct was verified by sequencing (Central Analytical Facility, Stellenbosch University). NCBI BLAST analysis was performed against the Arabidopsis genome to verify the sequence of the cloned gene.

Separation of DNA fragments by gel electrophoresis

All DNA isolations have been inspected on a 1% agarose gel, stained with 10 mg ml^{-1} ethidium bromide and visualized under UV light. Agarose gel experiments were conducted in 0.5X TBE buffer at a voltage of between 100 – 120V for at least an hour or until bands have separated sufficiently. TBE buffer (5X) contains 54 g L^{-1} Tris-base, 27.5 g L^{-1} boric acid and 20 ml L^{-1} 0.5 M EDTA (pH 8.0). Band sizes were determined with the help of a lambda *pst* ladder. DNA concentrations were determined on a NanoDrop 1000 (Thermo Scientific).

5.2.3 Competent yeast cells

Electro-competent yeast cells were made according to Becker and Guarente, 1991. The method entailed growing an overnight starter culture by inoculating a single yeast colony into 5 ml YPD media (10 g L^{-1} yeast extract, 20 g L^{-1} Peptone and 20 g L^{-1} dextrose) and shaking it at 240 rpm in a 28°C incubator. The starter culture was transferred to a 500 ml Erlenmeyer flask containing 200 ml YPD and was grown, as previously done, for 4 hours or until an OD_{600} 0.8 was reached. The culture was harvested at 4°C in a Sorvall® RC5C PLUS centrifuge at 5000 x *g* for 5 minutes. The pellet was gently resuspended in 10 ml of 10 x TE buffer (1 M Tris-HCl [pH 7.5] and 0.5 M EDTA [pH8.0]), followed by the addition of 10 ml lithium acetate (1 M) and shaken at 240 rpm for 45 minutes at a temperature of 28°C. Subsequently 2.5 ml dithiothreitol (1 M) (Sigma Alderich™) was added to the culture and shaken for another 15 minutes. The yeast cells were then washed three times in 50 ml ice cold water by centrifuging at 6000 x *g*. The pellet was resuspended in 25 ml ice cold sorbitol (1 M) and centrifuged at 6000 x *g*. The final pellet was resuspended in 500 µl sorbitol (1 M) and divided accordingly into 100 µl aliquots for the use in transformation.

5.2.4 Yeast transformation

Yeast cells were transformed using the Bio-Rad GenePulser Xcell™ electroporator according to manufacturer's instructions. The transformation mixture consisted of 100 µl electrocompetent yeast cells and 100 ng of DNA which was transferred to an ice cold electroporation cuvette with an aperture of 0.2 cm. Electroporation was carried out at pre-set protocols which included a pulse at 2.5 kV. The cells were recovered with ice cold sorbitol (1 M), incubated at 28°C for 30 minutes and 150 µl plated out onto selection media (see section 4.2.5).

5.2.5 Yeast growth and selection

Throughout the study, growth experiments of the yeast were conducted in YPD, YNB or M63 (Cohen and Rickenberg, 1956) media depending on the purpose of the experiment. The

yeast was grown at a temperature of 28°C and shaken at 240 rpm when in liquid. Growth of transformed yeast cells in the YNB and M63 media was carried out by supplementing the media with necessary amino acids which included 100 mM histidine-HCl, 100 mM leucine, 40 mM tryptophan, 10 mM adenine and 20 mM uracil. YPD media consisted of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} dextrose. Components of the YNB media included 6.6 g L^{-1} YNB (yeast nitrogen base), 20 g L^{-1} glucose (filter sterilised through a 0.2 µm filter), amino acids to desired concentrations and brought to a pH of 5.6 with the addition of concentrated HCl. YPD and YNB media were solidified with 20 g L^{-1} agar when necessary. M63 was utilised as minimal media (Clifton *et al*., 1978) due to the ease of supplying phosphate to media in various concentrations as required. The M63 media consisted of 13.6 g L^{-1} KH_2PO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 mg L^{-1} $FeSO_4$-$7H_2O$, adjusted to pH 5.6 and autoclaved. After sterilisation the M63 media was supplied with filter sterile 1 ml L^{-1} $MgSO_4$-$7H_2O$ (1 M) and 0.2 % v/v glucose. In the event of conducting complementation studies the M63 media was adjusted slightly. KH_2PO_4 was replaced with KCL and the media was supplied with various phosphate concentrations ranging from 0 to 600 µM. Selection depended on the plasmid pHVX2, which carries a *Leu*2 gene, enabling transformants to grow successfully without supplementation of leucine which is a prerequisite amino acid for the PAM 2 yeast strain.

Putative yeast transformants were verified with the yeast plasmid extraction protocol using zymolase and a manifold miniprep method. The procedure included the growth of yeast in 1 ml selection media (YNB without leucine) until the culture reached saturation. The culture was harvested at 13 000 x *g* for one minute at room temperature in a desktop microfuge (Biofuge, Heraues). The pellet was resupended in 100 µl of 67 mM KH_2PO_4 (pH 7.5), followed by the addition of 10 µl driselase (25 µg $µl^{-1}$ dissolved in 10 mM Tris buffer, pH 7.5) (Sigma®) and incubated for one hour at 37°C. The incubation step was followed by the addition of 200 µl lysis solution (1 ml SDS [10% w/v], 200 µl NaOH [10N in 10 ml water]) and 200 µl neutralizing solution (4M KoAc, pH 5.5). The suspension was centrifuged for 10 minutes at 13000 x *g*, passed through a miniprep column (Fermentas®) and eluted in 25 µl of sterile water. The DNA was subsequently transformed back into *E.coli,* as described in section 4.2.4, and transformants were verified via colony PCR with the following primers, fw: 5'-CAAGATTTTCTCTAGAGTGACTGAACAAC-3' and rev: 5'-GAGTAACACAAAATAATTCTAGAGGG

ACTTTTCTACCGG-3'. The PCR reaction mixture contained 10 µM of each primer, 10 mM dNTPs, 25 mM $MgCl_2$, 0.5 units of polymerase and approximately 100ng DNA. The PCR setup were as follow, an initial denaturation step at 95°C for 2 minutes followed by 35 amplification cycles (denaturation at 98°C for 20 seconds, annealing at 55°C for 15 seconds and an extension at 72°C for 1 minute), a final extension at 72°C for 2 minutes.

5.3 Results and Discussion

5.3.1 Gene propagation and analysis of transgene presence

The *PHT*1;5 gene was cloned into the 2micron multi-copy plasmid, pHVXII, which contain a yeast promoter and terminator for effective foreign gene transcription. This is an *E.coli*-yeast shuttle vector which enables gene propagation in a bacterial system as well as a yeast system. The presence of the transgene was verified by means of colony PCR and restriction enzyme digest. PCR fragments were analysed on a 1% agarose gel (Figure 24: A) and expected sizes of 1.7 kb was observed. To further confirm the presence and orientation of the transgene in the correct vector, restriction digests were performed (Table 4). The presence of the gene was tested for by digesting the DNA on four respective occasions with different enzymes (Table 4, shaded rows) and the orientation was analysed by means of digesting the DNA with *Sma*I and *EcoR*I.

Table 4: Expected fragment sizes for pHVXII and pHVXII-*PHT*1;5 after restriction digests with specific enzymes

	Fragment sizes expected from digests (bp)	
Enzymes	**pHVXII**	**pHVXII-*PHT*1;5**
***Bam*HI**	7500	9200
***Bgl*I+*Sca*I**	7500	4300, 4900
***Kpn*I**	7500	7400, 1800
***Xho*I**	7500	8100, 1100
***Sma*I**	7500	9200
***Eco*RI**	7500	7700, 900, 600

All digests were analysed on 1% agarose gels and confirmed the presence (Figure 24: B) and correct orientation (Figure 24: C) of *PHT*1;5 in the plasmid. Successful gene propagation was carried out for the yeast constructs and authenticity and correct frame of the gene was further verified by sequencing and BLAST analysis against the *Arabidopsis* genome. Sequence data confirmed the correctness of the gene and indicated that no mutations were present in the sequence.

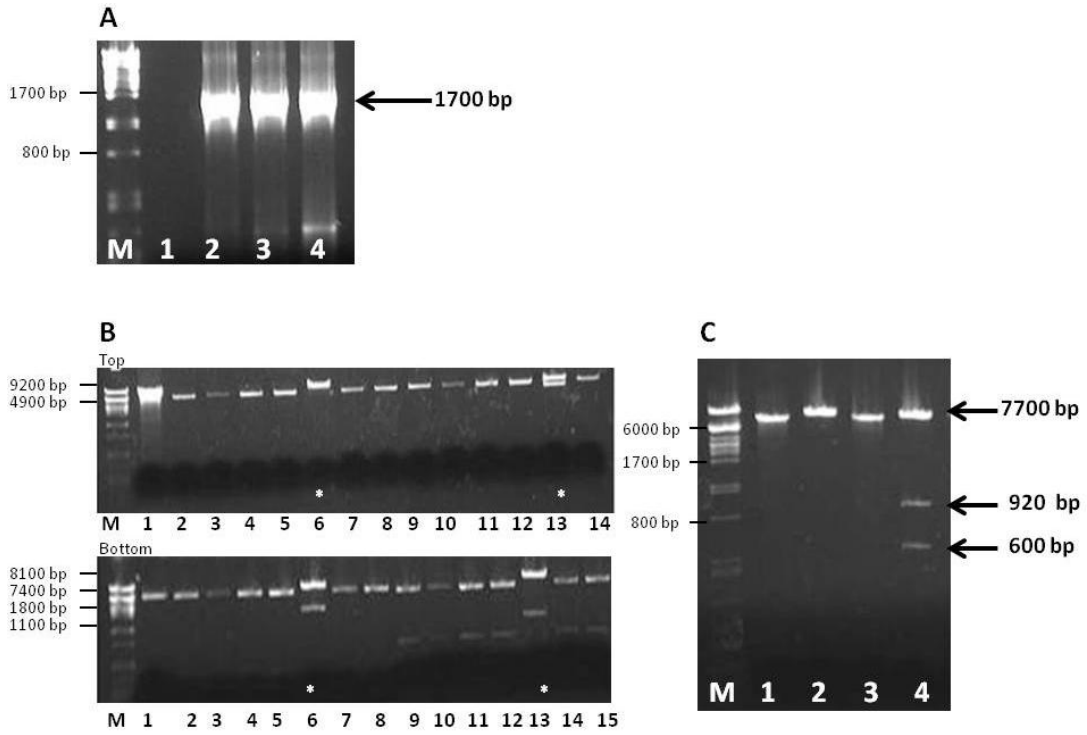

Figure 24: (**A**) Colony PCR with gene-specific primers of *PHT*1;5 in pHVXII. Lanes, M – Molecular marker Lambda *pst*I, 1 - Negative water control, 2 - Positive gene control, 3 – positive colony one, 4 – positive colony two. (**B**) Seven colonies tested for containing pHVXII-*PHT*1;5 construct and analysed with restriction digestion (correct colony indicated with asterix). (**B: Top**) Lanes, M – Molecular marker Lamda *pst*, 1 - empty pHVX2 (*Bam*HI), 2- 8 digest with *Bam*HI (colonies 1 to 7), 9 – 14 digest with *Bgl*II+*Sca*I (colonies 1 to 6) (**B: Bottom**) Lanes, M – Molecular marker lamda *pst*, 1 – Colony 7 digest with *Bgl*II+*Sca*I, 2 – 8 digest with KpnI (colonies 1 to 7), 9 – 15 digest with *Xho*I (colonies 1 to 7). (**C**): Orientation check of correct colony. Lanes: M – molecular marker Lamda *pst*, 1 - empty pHVX2 (*Sma*I digest), 3-pHVX2-*PHT*1;5 construct (*Sma*I digest), 4-Empty pHVX2 (*Eco*RI digest), 5-pHVX2-*PHT*1;5 construct (*Eco*RI digest)

Yeast transformations were successfully conducted via an electroporation method and putative transformants were selected for by supplying the growth media with necessary amino acids. Once putative transformants were obtained the plasmid DNA had to be isolated from the yeast and transformed back into *E.coli* for verification. This back-transformation step was necessary because yeast display a low copy number multiplication of plasmids. This low plasmid copy number in yeast is often not readily detected when yeast colonies are analysed, resulting in false conclusions. During this experiment it was demonstrated that yeast was successfully transformed with the pHVXII-*PHT*1;5 construct as well as a control (empty pHVXII). Positives clones were subject to further complementation studies.

5.3.2 Yeast Complementation

In the event of starting the complementation trials, it was found that the PHT1;5 did at first not seem to complement any growth defect of the PAM2 strain. In fact, the strain seemed to grow without restraint on selective minimal yeast synthetic dropout (SD) media when supplied with either minimal amount of phosphate or without phosphate (Figure 25). According to literature, this was the standard media that had been utilised during complementation studies of other plant phosphate transporters in yeast (Guo,2008; Versaw and Harrison, 2002; Daram *et al*., 1999). Most of these characterised transporters however, are low-affinity transporters with a relative high Km value for phosphate usually in the milli molar range.

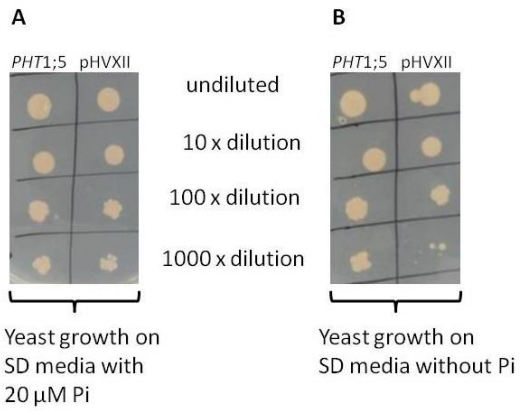

Figure 25: Serial dilutions of yeast transformants growing on synthetic dropout media supplied with (**A**) 20 µM phosphate or (**B**) no phosphate. Cultures were spotted onto media in equal volumes

It was speculated that the media used for studying the complementation with the PHT1;5 transporter contain traces of phosphate contamination which would therefore lead to false results. A different media was thus formulated for this study and it was based on the M63 minimal media, with minor modifications. The modified media contained equivalent concentrations of KCl rather than KH_2PO_4 and yeast was selected for by supplementing the media with necessary amino acids.

The ability of PHT1;5 to complement the defective yeast strain was tested for on the modified M63 media by investigating growth rescue when different phosphate concentrations was supplied. During this study it was demonstrated that the PHT1;5 transporter is able to rescue the growth of the PAM2 yeast strain on phosphate concentrations as low as 25 µM (Figure 26: A). This experiment was also conducted successfully with phosphate concentrations of 50 µM (Figure 26: B), 100 µM (Figure 26: C) and also on M63 media that was supplied with the full phosphate concentration of 100 mM (Figure 26: D). These results clearly indicate that the PHT1;5 transporter was able to mediate the transport of inorganic phosphate into yeast cells at very low concentrations.

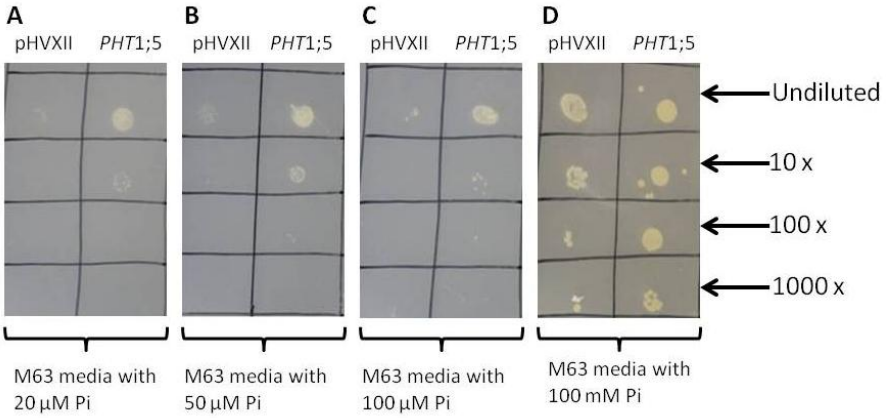

Figure 26: Serial dilution of yeast transformants spotted onto M63 minimal media in equal volumes. On each plate is cultures harbouring either the control (empty pHVXII) which can be observed in the left lane or the transformed (pHVXII-*PHT*1;5 construct) on the right (**A**) Minimal media supplied with 25 µM phosphate, (**B**) Minimal media supplied with 50 µM phosphate, (**C**) Minimal media supplied with 100 µM phosphate, (**D**) Minimal media supplied with 100 mM phosphate

This evidence, in combination with the fact that growth occurred in the control at higher phosphate concentrations, confirms, at least in part, the speculation that PHT1;5 is a high-affinity phosphate transporter. It cannot be ignored that the possibility exists for this transporter to directly or indirectly work alongside, or up-regulate, an endogenous transport mechanism of yeast. Although this complementation on M63 minimal media was consistent, we can only speculate that the Km range for this transporter is approximated to be in the range of 20 µM as this was where strongest complementation was displayed on numerous

occasions. It would be necessary in future studies to investigate transport kinetics by determining the true Km value for this transporter. This would provide conclusive evidence that the Pi transport ability is associated with the PHT1;5 protein exclusively and that there is no interference from endogenous yeast transport mechanisms.

5.3.3 Concluding remarks and future prospects

Yeast has been skilfully utilised over the years for studying membrane transporters from plants due to its relative rapid growth rate and the fact that is able to perform post-translational modification. Not only does it display advantages of expressing eukaryotic proteins, but some mutant strains of yeast have been developed that display deficiencies in transport pathways (Riesmeier *et al*., 1992). These mutant strains enable researchers to successfully conduct complementation studies and is useful for assigning possible functions to uncharacterised plant transporters.

In order to investigate the hypothesis that PHT1;5 is a high affinity transporter, we tested its ability to complement the growth defect of the mutant yeast strain PAM2. This strain does not harbour either of its natural high-affinity phosphate transport systems, PHO84 and PHO89, and therefore result in reduced growth during conditions of phosphate limitation. The strain does, however, still harbour the low-affinity transport systems enabling ample activity during non-selective growth conditions for efficient propagation.

Results obtained during this study provides evidence of the biochemical function of the PHT1;5 transporter to mediate phosphate transport. It has been revealed via complementation of the yeast mutant strain, PAM2, that PHT1;5 is able to functionally transport inorganic phosphate when grown on minimal media supplemented with extremely low concentrations of phosphate. It is evident that this eukaryotic expression system can be successfully used for the characterisation of PHT1;5 in future studies. During these experiments we were able to demonstrate the ability of PHT1;5 to function as a high-affinity

phosphate transporter, because the growth defect of the PAM2 strain was restored during growth conditions of extreme low Pi concentrations. This was clearly demonstrated when PAM2 harbouring the empty vector (pHVXII) grew only at concentrations higher than 100 µM, while cells expressing *PHT*1;5 was able to grow at Pi concentrations as low as 25 µM. The growth rate of the mutant strain containing the empty vector (pHVXII) correlated positively with increasing phosphate concentrations, its growth was limited at micro-molar concentrations. All strains, empty PAM2, PAM2-pHVXII and PAM2-pHVXII-*PHT*1;5, grew well at milli-molar phosphate concentrations due to the internal low-affinity Pi-uptake system of yeast being active (Tamai *et al*.,1985).

CHAPTER 6

General discussion

There were three main objectives set out for this study. Firstly, to analyse the protein topology and gene expression profile *in silico*. Secondly, to utilise mutant *E.coli* and yeast strains as tools for heterologous expression and to complement the growth defects of these strains with the PHT1;5 transporter. Thirdly, to determine whether PHT1;5 is a high- or low-affinity phosphate transporter. All together the aim of the study was to participate in the characterisation process of PHT1;5.

In silico analysis of PHT1;5 revealed that this protein is composed of 542 amino acid residues and that it is generally hydrophobic, indicating its membrane bound association. It is hypothesised that PHT1;5 is targeted to the chloroplast inner membrane, although weak evidence supports this statement. Programs such as ChloroP and TargetP predicted that there is no strong evidence for PHT1;5 being targeted to the chloroplast, although it did reveal the presence of a putative chloroplast transit peptide. Some previous studies done to investigate this provides no definite insight as to where the protein is targeted to, but merely states that it is highly expressed during conditions of Pi depletion (Zwiegelaar, 2010; Nagarajan *et al*., 2011). Zwiegelaar (2010) conducted *PHT*1;5-GFP fusion studies and demonstrated that the protein is being targeted to the chloroplast but these results would need further confirmation by performing studies on isolated chloroplasts rather than on whole plant level.

The expression profile for *PHT*1;5 revealed consistent results. It was predicted to be expressed mainly in the aerial parts of the plant which can lead to the assumption that it is less likely to play a role in the transport of phosphate from soil. It should, however, be noted that these expression profiles are predicted for growth during conditions of optimal nutrition and not for phosphate depleted conditions. A recent study done by Nagarajan and colleagues (2011) revealed that *PHT*1;5 is also expressed in the roots during phosphate limitation, even though these findings were not confirmed when Zwiegelaar (2010) investigated the expression profile of the gene during phosphate limiting conditions. It is

therefore suggested that further expression analysis need to be conducted to fully understand the transport and nutritional condition profile for *PHT*1;5. The statement by Himelblau and Amasino, 2001, declared that approximately 78% of Pi stored by the older parts of a plant is transported to younger tissues in order to mediate an adequate Pi homeostasis. This might emphasize the involvement of PHT1;5 in nutrient mobilisation and implies the possibility of this transporter to display characteristics of both Pi import and export.

During the *in silico* analysis it was found that PHT1;5 is part of the major facilitator superfamily (MFS) of Pi/H^+ transporters with a large proportion of the protein being conserved throughout similar transporters from different organisms. The sequence was aligned to known high affinity transporters such as PHO84 from *Saccharomyces cerevisiae* (Bun-Ya *et al*., 1991), PHO5 from *Neurospora crassa* (Versaw, 1995) and GvPT from *Glomus versiforme* (Harrison and Van Buuren, 1995) and it was revealed that there was some sequence similarity, especially one conserved region that included a phosphate permease. The sequence analysis indicated, based upon similarity, that PHT1;5 is indeed a inorganic phosphate transporter. In the event of investigating the amount of transmembrane helices (TMs), ambiguous predictions were found and it was decided to assume that PHT1;5 has 12 TMs based on the characteristics revealed in MFS. Sequence analysis of the Pht1 family of transporters in Arabidopsis indicated high similarity between all nine members, including PHT1;5.

Escherichia coli is a well-established heterologous expression systems (Frommer and Ninnemann, 1995) and displays several advantages above other systems. These advantages include simplicity to work with, rapid growth rate, high yield of protein production and relative low expense. Genetic and biochemical information about this prokaryote is readily and extensively available, various strains and mutants are easy to come by and all commercially available vectors are compatible for *E.coli* transformation. For these reasons, *E. coli* was investigated during this study for its ability to serve as a heterologous expression system for the plant transporter PHT1;5. Previous research have identified *E.coli* as being a successful heterologous expression tool (Kuhlbrandt and Wang, 1991; Kim *et al*., 1998; Uozomi *et al*.,1998), but these studies seem to be negligible.

During our investigation, *E.coli* experiments seem to be mostly inconclusive or unreliable and it is therefore not suggested as choice heterologous expression system for membrane bound transporters. Membrane bound proteins often induce growth arrest and to have toxic effects on bacterial cultures (Yin *et al*., 2007) and we suspect that induction of the PHT1;5 protein display these toxicity effects. These toxic effects might also be interpreted as being a result of the possible Pi exporting nature displayed by PHT1;5 which would then lead to a constitutive export of Pi from the bacterial cells, leading to an extensive Pi starvation period after induction. This statement is based upon the assumption that low protein levels might be present in the bacterial system but was not visible in total protein extracts. Future studies might include the optimization of growth conditions and the use of different *E.coli* strains when membrane bound proteins are to be investigated. Strains such as BL21-CodonPlus-RIL or BL21-CodonPlus-RP cells are recommended by Stratagene® to be used during studies where proteins are potentially toxic to bacterial cultures.

Inconsistent and inconclusive data was obtained from the *E.coli* complementation studies. Although *E.coli* could not be effectively utilised as heterologous expression system during this study, it is still proposed that the PHT1;5 transporter function as a H^+/Pi symporter due to the immediate change observed when pH was measured. A plausible reason for this might be that the PHT1;5 transporter, regardless of it being toxic when induced, might have a rapid functionality in phosphate stressed bacterial cultures where it imports Pi ions in exchange for H^+ ions.

During this study yeast has proven to be a successful heterologous expression system in that the growth defect of the PAM*2* strain was complemented for by the PHT1;5 transporter during conditions of low Pi availability. Here we provide some insight as to whether PHT1;5 is a high- or low-affinity Pi transporter. It was found that, during extremely low Pi concentrations, PHT1;5 was able to function as a high affinity transporter and functionally transport Pi across the yeast plasma membrane. Evidence gained during these experiments provides only a relative range of phosphate concentrations where PHT1;5 is active, but does not present the Km value of this transporter. Complementation of yeast cells were achieved at Pi concentrations as low as 25 µM and it can therefore be speculated that the Km value

for PHT1;5 is in concordance with other high affinity transporters, displaying values of between 1-40 µM (Leggewie *et al*., 1995; Daram *et al*., 1999). However, it should be taken into consideration that previous studies have indicated that yeast heterologous expression does not always lead to accurate estimations of Km values for plant transporters (Leggewie *et al*., 1995). Leggewie *et al*. (1995) investigated the LePT1 phosphate transporter from tomato and was able to complement the PAM*2* yeast strain. They indicated via radio-active analysis that the transporter displayed a Km value of 31 µM which was, according to Raghothama (1999), an improvement of previous results but still too high for physiological high-affinity transport on a whole plant level. When the Arabidopsis homolog of LePT1 was characterised in cultured tobacco cells its Km value was 3.1 µM (Mitsukawa *et al*., 1997). These statements indicate that yeast has some limitations when kinetic properties of plant transporters need to be accurately estimated and should therefore be used only as putative values.

Future analysis of PHT1;5 and its transport activity should include the determination of the Km value through either radio-active labelling or by utilising the newly developed nanosensor technology. Radio-active labelling has been successfully used in previous studies to determine Km values of plant transporters (Leggewie *et al*., 1997; Daram *et al*., 1998; Daram *et al*., 1999; Guo, 2008). The nanosensor technology makes use of fluorescence resonance energy transfer (FRET) and the nanosensors are based on conformational changes of a fluorescent indicator protein (FLIP) in response to changes in metabolite concentrations. This technology has some advantages over radio-active labelling, such as the ability to analyse metabolite presence within subcellular compartments and at very low concentrations and would enable researchers to investigate this transporter in more depth *in planta*. Several FRET Pi sensors (FLIPPi), varying in sensitivity, have been developed and characterised (Gu *et al*., 2006). Furthermore, PHT1;5 should be characterised by determining its optimum pH range and also by exploiting the effect that various other chemical compounds might display on its Pi transport functionality.

Based upon the prediction, despite the fact that weak evidence supports this, that PHT1;5 is localised to the chloroplast inner membrane, we believe that this transporter is a participating candidate in the complex Pi regulation and transport machinery in higher

plants, although these activities are poorly understood in the plant cell (Neuhaus and Maass, 1996; Neckelmann and Orellana, 1998). For future endeavours, it would be of significant value to investigate the underlying Pi acquisition properties of plant cells by modifying the biochemical properties of Pi transporters/transport systems. Studies based upon this might lead to insightful information with regards to how plants develop under Pi limiting conditions, how photosynthesis and carbon partitioning is affected and how stress signalling pathways interact with Pi transport pathways (Marschner, 1995; Hurry *et al*., 2000). Future concepts regarding the development of PHT1;5 over expression on plants should, however, take into consideration that Pi in too high concentrations might become toxic to the plant. This might highlight the possible reason for why Pi uptake and transport is regulated in such a complex manner. A clearer understanding of how plants perceive and control intracellular signalling networks in response to wavering Pi conditions is thus a critical step toward understanding how plants regulate and maintain Pi homeostasis and metabolism during Pi depletion.

The eventual goal for characterising these plant Pi transporters would be to generate high yielding plants that are able to effectively grow on Pi-poor soil without the supplementation of fertilizers. Therefore it can be believed that a comprehensive understanding of Pi acquisition and distribution by plants will have a significant impact on environmental and agricultural concerns, especially with regards to crop development.

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