MOLECULAR STUDIES ON PHOSPHATE HOMEOSTASIS IN HIGHER PLANTS

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date:

Summary

Phosphorus (P) is essential for the survival of all living organisms and forms part of several key biological molecules and processes. The basic biological function of all cells depends on the availability of P as structural element in phospholipids and nucleic acids. P plays a central role in the energy metabolism of the cell by activating metabolic intermediates of carbohydrate metabolism and by acting as an energy currency in the form of adenosine tri-phosphate (ATP). ATP is produced during photosynthesis from the energy derived from sunlight, probably the most important biological process on earth. The balance of P supply and demand is of critical importance here. Plants assimilate P in the form of orthophosphate (P_i) via its roots and utilises complex mechanisms to redistribute and balance the P_i concentrations throughout the plant. These processes are collectively known as phosphate homeostasis and in this study we utilised molecular techniques to study some key aspects of this complex network of mechanisms in the plant *Arabidopsis thaliana*.

When the role of the PHT1;5 P_i transporter was investigated in photosynthesis under P_i limitation a new mechanism utilised by plants to supply P_i for the production of ATP in the chloroplast was discovered. During periods of adequate P_i supply plants make use of the triose phosphate / phosphate translocator (TPT) to exchange P_i for phosphorylated carbon intermediates. This transporter does, however, not function at the low P_i concentrations present during P_i limitation and the plant therefore express an alternative transporter *i.e.* PHT1;5. Together with this transporter several genes were identified that was expressed to allow the export of carbon intermediates from the chloroplast via starch turnover. Amongst these, several alternative isoforms of the enzymes responsible for starch turnover are expressed during P_i limiting conditions. It is therefore suggested that the products of starch degradation, e.g. glucose and maltose are the potential candidates for carbon export from chloroplasts under P_i limiting conditions.

In an attempt to perturb the P_i concentrations in the *Arabidopsis* vacuole we expressed the three genes of a newly discovered polyphosphate (PolyP) polymerase from the yeast *Sacharomyces cerevisiae* in *Arabidopsis*. This enzyme complex accumulates PolyP in the yeast vacuole and since the plant vacuole is playing a key role in buffering P_i concentrations we anticipated some observable effects that could lead to the elucidation of the mechanisms involved. Production of PolyP was conclusively shown in plant callus, but it was only at very low concentrations with no detectable perturbing effect and undetectable in whole plants.

With the aim to apply this technology to the PolyP and PHT1;5 lines developed in the other parts of this study, newly developed fluorescent indicator protein nanosensors ($FLIPP_i$) were evaluated as a method for detecting and monitoring *in vivo* P_i concentrations in multicellular plant organs. This technique is

capable of detecting changes in metabolite concentrations in real-time and it was applied to the roots of *Arabidopsis* seedlings subjected to P_i limitation. We specifically looked at changes in the cytosol, but our results revealed no detectable changes occurring in the P_i concentrations in this compartment. This was interpreted to indicate lower levels of P_i in this compartment as was previously expected.

Opsomming

Fosfaat (P) is essensieël vir die oorlewing van alle organismes en maak deel uit van etlike kern biologiese prosesse en molekules. Die basiese biologiese funksionering van alle selle hang direk af van die beskikbaarheid van P as strukturele element van fosfolipiede en nuklëinsure. Fosfaat speel 'n sentrale rol in die energie metabolisme van 'n sel deur metaboliese intermediante te aktiveer en deur op te tree as die geld eenheid van sellulere energie in die vorm van adenosien tri-fosfaat (ATP). ATP word gegenereer gedurende fotosintese vanaf die energie wat van sonlig vasgevang word, dit is waarskeinlik die belangrikste biologiese proses op aarde. Dit is van kritiese belang dat die fosfaat vraag en aanbod hier fyn gebalanseer word. Plante assimileer P in die vorm van ortofosfaat (P_i) deur hulle wortels en maak gebruik van komplekse meganismes om P_i deur die plant te versprei en konsentrasies te balanseer. Hierdie prosesse staan gesamentlik bekend as fosfaat homeostase en in die huidige studie het ons gebruik gemaak van molekulêre tegnieke om 'n paar belangrike aspekte van hierdie komplekse netwerk van prosesse in die plant *Arabidopsis thaliana* te bestudeer.

Toe die rol van die PHT1;5 P_i transporter in fotosintese onder toestande van P_i tekort bestudeer is, is 'n nuwe meganisme ontdek waarmee plante P_i verskaf aan chloroplaste vir die proses van fotosintese onder toestande van P_i tekort. Gedurende periodes wat die plant genoegsame P_i tot sy beskikking het, word van die triose fosfaat / fosfaat uitruiler (TPT) gebruik gemaak om P_i uit te ruil vir gefosforileerde koolstof metaboliete. Hierdie transporter kan egter nie onder die lae P_i konsentrasies wat voorkom in die sitoplasma onder P_i tekort toestande funksioneer nie, en gevolglik moet die plant van 'n alternatiewe transporter naamlik PHT1;5 uitdruk. Verskeie ander gene is ook geidentifiseer wat saam met hierdie transporter onder toestande van P_i tekort uitgedruk word en die plant toelaat om koolstof tussengangers uit die chloroplaste uit te vervoer via die proses van stysel produksie en afbraak. Onderandere is verskeie alternatiewe isoforme van die gene wat verandwoordelik is vir stysel produksie en afbraak identifiseer wat uitgedruk word onder toestande van P_i tekort.

In 'n poging om die P_i konsentrasies in die *Arabidopsis* vakuool te versteur is drie gene van die nuut ontdekte polifosfaat (PolyP) polimerase kompleks van die gis *Sacharomyces cerevisiae* in *Arabidopsis* uitgedruk. Hierdie ensiem kompleks is verandwoordelik vir die akkumulasie van PolyP in die gis vakuool en siende die plant vakuool 'n kern rol speel in die buffering van P_i konsentrasies in die plant, het ons sekere waarneembare gevolge verwag wat kon lei tot die ontrafeling van die meganismes hierby betrokke. Die produksie van PolyP in plant kallus is duidelik gedemonstreer, maar dit was slegs teen baie lae konsentrasies met geen waarneembare versteuringseffek nie, en kon glad nie in heel plante waargeneem word nie.

Met die oog daarop om hierdie tegnologie toe te pas op die bestudering van die PolyP en PHT1;5 lyne wat in die ander dele van hierdie studie ontwikkel is, is 'n nuut ontwikkelde fluoresente indikator protein nanosensor (FLIPPi) tegnologie evalueer as 'n metode om P_i konsentrasies *in vivo* in multisellulere plant organe waar te neem en te monitor. Hierdie tegniek is in staat daartoe om veranderinge in P_i konsentrasies in selle direk te monitor en is gevolglik op die wortels van *Arabidopsis* saailinge onder P_i tekort toestande toegepas. Daar is spesifiek na veranderinge in die sitosol gekyk, maar ons resultate kon geen waarneembare veranderinge in P_i konsentrasies in hierdie kompartement uitwys nie. Hierdie resultaat beteken waarkeinlik dat die P_i konsentrasies in hierdie kompartement waarskeinlik baie laer is as wat voorheen verwag is.

"The most incomprehensible thing about the world is that it is comprehensible."

- Albert Einstein -

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Preface

This dissertation consists of **6 chapters** of which **Chapters 3, 4** and **5** contain the bulk of the experimental work performed during this study. These chapters were prepared as research articles to be submitted to individual journals for publication. The dissertation as a whole was written in a single citation style except for **Chapter 3** that will be submitted for publication to the Nature journal and was therefore written in the style of this journal that differs greatly from the default. **Chapter 1** was written as a general introduction to the whole dissertation, explaining the logic and reasoning behind the research, the objectives of the various chapters and how they are linked. **Chapter 2** gives the reader some insight into the various research fields and subjects covered throughout this study, by providing some essential background and explaining the importance of certain aspects. In **Chapter 6** the findings of the research chapters are summarised by emphasising the importance of the major discoveries and how they all tie into the bigger picture of phosphate homeostasis in plants.

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Abbreviations

APS2	ADP-glucose pyrophosphorylase 2
BAM5	β-amylase 5
BASTA	Herbicide used as selection agent in plant transformation
BMY1	β-amylase 5
^{14}C	Radioactive carbon 14
CFP	Cyan fluorescent protein
CHO	Chinese hamster ovary cells
DPE	Disproportionating enzyme
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
EYFP	Enhanced yellow fluorescent protein
FLIM	Fluorescence lifetime imaging
FLIPPI	Fluorescent indicator protein for the detection of phosphate
FLIPS	Fluorescent indicator proteins
FRET	Fluorescence / Förster resonance energy transfer
F_V/F_M	Maximum quantum yield of PSII
GFP	Green fluorescent protein
GPT	Glucose 6-phosphate/Pi translocator
GV3101	*Agrobacterium tumefaciens* strain
GWD	Glucan-water-dikinase
IPS/AT4	Non-coding RNAs acting as ribo regulators to control the levels of MicroRNA 399
MDP	Methylene diphosphonate
MES	2-(N-morpholino)ethanesulfonic acid
MIR399	MicroRNA 399
MIRNA	MicroRNA
MOPS	3-(N-morpholino)propanesulfonic acid
MS	Murashige and Skoog media for plant growth
NPQ	Non-photochemical quenching
P	Phosphorous
PAGE	Polyacrylamide gel electrophoreces
PBPS	Periplasmic binding proteins
PHM1 / 2 / 3 / 4	Genes for the subunits of the polyphosphate polymerase in yeast
PHO	Phosphate regulatory pathway in yeast

PHO2	Phosphate accumulation mutant 2
PHT	Phosphate transporter
Pi	Orthophosphate
PolyP	Polyphosphate
pPT	Plastidic phosphate translocator
PPT	Phosphoenolpyruvate/Phosphate translocator
PPX	Exopolyphosphatase
PT	Phosphate translocator
qP	Photochemical quenching parameter / Proportion of open PSII
RAM1	β-amylase 5
RFP	Red fluorescent protein
ROI	Region of interest
TPT	Triose phosphate/phosphate translocator
Tris	Tris(hydroxymethyl)aminomethane
UBC24	Ubiquitin conjugase-related enzyme 24
XPT	Xylulose 5-phosphate/Pi translocator
YFP	Yellow fluorescent protein
Φ_{PSII}	Quantum yield of PSII

-Chapter 1-

General introduction

The importance of Phosphorus (P) as a nutrient for all living organisms, together with the relatively low availability of this mineral in soil (1, 2), have resulted in the evolution of extremely diverse and complex mechanisms for its uptake and distribution (3, 4). These processes employed by organisms to assimilate, store and distribute P, as well as the complex mechanisms utilised to balance supply and demand are in general referred to as phosphate (P_i) homeostasis (3). The importance of P_i in the normal growth and development of plants necessitates well developed homeostatic control mechanisms by the plant in order to tightly control the concentrations of this critical metabolite in all compartments (3). It is no wonder then that P_i acquisition, endogenous P_i pool sizes and P_i exchange between compartments have been the focus of many studies (4, 1, 5-8). The present study focused on a few key questions surrounding P_i homeostasis in plants.

The process of maintaining P_i homeostasis is extremely complex and although it is one of the most important aspects of biological systems and directly impacts on the productivity of cultivated plants, it is still poorly understood. An overview of the current understanding of P_i homeostasis and phosphate transport is discussed in Chapter 2. The sheer complexity and amount of factors involved in the balance of P_i supply and demand makes it difficult to study the entire process. We therefore focussed our investigation on specifically selected aspects surrounding P_i homeostasis in order to shed some light on the overall picture.

One important aspect adding to the complexity of P_i homeostasis is that P_i is important across all sub-cellular compartments and that the role and concentration of P_i in each of these compartments varies depending on the compartment in question (9). This makes its study extremely difficult. P_i transporters are the proteins largely responsible for maintaining the sub-cellular balance of this anion. To date, five families of P_i transporters have been identified in plants and each family has several members with different functions (10-12). Membrane proteins, and in particular transporters, are difficult to study because their characterisation requires isolation of intact sub-cellular compartments, which is not always possible or in many cases unreliable. Techniques that do not require invasive extraction procedures, such as ^{31}P NMR, are again limiting in sensitivity and can only distinguish between compartments with varying pH. Background information on techniques that can be used to study intracellular metabolite concentrations is discussed in Chapter 2.

This area of P_i homeostasis, namely membrane transport, as well as the measurement of P_i concentrations in various sub-cellular compartments were investigated in this study. The adaptation of plants to P_i limitation is one of the most astounding parts of plant metabolism. We investigated this phenomenon by characterising the role of PHT1;5, one of the high affinity transporters in the PHT1 family that has previously been implicated in P_i redistribution and P_i homeostasis between different parts of the plant. The role of this transporter in photosynthesis under P_i limitation was investigated (Chapter 3).

With the hope of identifying some of the mechanisms utilised by the plant in balancing P_i concentrations we aimed to study the effect of creating an additional P_i pool in the plant vacuole. A yeast enzyme complex consisting of 3 genes responsible for the production of polyphosphate (PolyP) in the yeast vacuole (13) was expressed in *A. thaliana* (Chapter 4).

In order to understand P_i homeostasis in plants, accurate measurement of sub-cellular concentrations is needed and the newly developed nanosensor technology, where Förster / Fluorescence resonance energy transfer (FRET) is utilised to determine intracellular concentrations of metabolites was applied to measure P_i concentrations in *Arabidopsis* plants (14). Sensors specifically developed for the detection of P_i, previously characterised in mammalian cells, were evaluated for their use in plants with the specific aim of applying this technology to the genetic variants created in the other chapters (Chapter 5).

The observations and discussions of Chapters 3-5 are integrated in Chapter 6 and there, amongst others, possible future research aims to elucidate this very important yet complex process of P_i homeostasis is highlighted.

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-Chapter 2-

General background

Phosphate homeostasis

Phosphorous (P) forms an integral part of most cellular processes and the metabolism in all living organisms. It is utilised as structural and functional component of all cells as part of phospholipids in cellular membranes. It forms the structural backbone of all nucleic acids and acts as the major 'energy currency', in the form of ATP and ADP, used to transfer and store energy in the cell. P also plays a central role in the regulation of cellular signalling cascades as substrate for kinase and phosphatase reactions. For these reasons it is crucial that the cell regulates the concentration of phosphate by controlling its uptake and storage as well as the redistribution amongst the various sub-cellular compartments. This process is known as phosphate homeostasis and in essence describes the mechanisms that the organism in question applies in order to maintain this very delicate balance.

These statements hold true for all known organisms including Higher Plants, which are in the focus of the present study. Higher Plants, however, need to deal with a few unique problems. Amongst all the minerals needed by the plant to survive and function properly, P is probably the least available for uptake in the soil. The total P content of soil can be high, but plants are sensitive to the form of P that it can assimilate and need P to be in the form of inorganic P or orthophosphate (P_i) for it to be effectively assimilated. This form of P is present in very low concentrations in soil, usually lower in concentration than other macronutrients (1) and is seldom found at concentrations above 10µM and can drop to below that at the root/soil interface (2). For this reason plants needed to evolve some unique developmental and metabolic mechanisms to deal with P_i deficiency. These adaptations are aimed at the optimisation of P_i acquisition from the soil and to help plants to survive under low P_i availability (3, 4).

The distribution of P_i in the soil is also very heterogeneous and results in certain behavioural patterns in root growth recently reviewed by Desnos (5). The distribution of P_i in the plant is also heterogeneous and is tightly regulated according to physiological demand. When P_i is limiting, shoots accumulate more P_i than roots and metabolically active cells more than inactive cells. Subcellularly, excess P_i is accumulated largely in the vacuole and has higher levels in the plastids than the cytoplasm (2, 6, 7). Sophisticated regulatory mechanisms are required by the plant in order to maintain such an unbalanced distribution of P_i. During non-equilibrium conditions when acquisition rates vary, the plant needs to maintain all sub-cellular and intracellular distribution patterns in such a way that metabolism functions at an optimal rate.

A recent review by Doerner (2008) provides an excellent overview of a proposed signalling module that controls phosphate homeostasis in the plant system and covers our understanding of the mechanisms involved in the regulation of P_i homeostasis and also highlights the (numerous) gaps in our current knowledge (8).

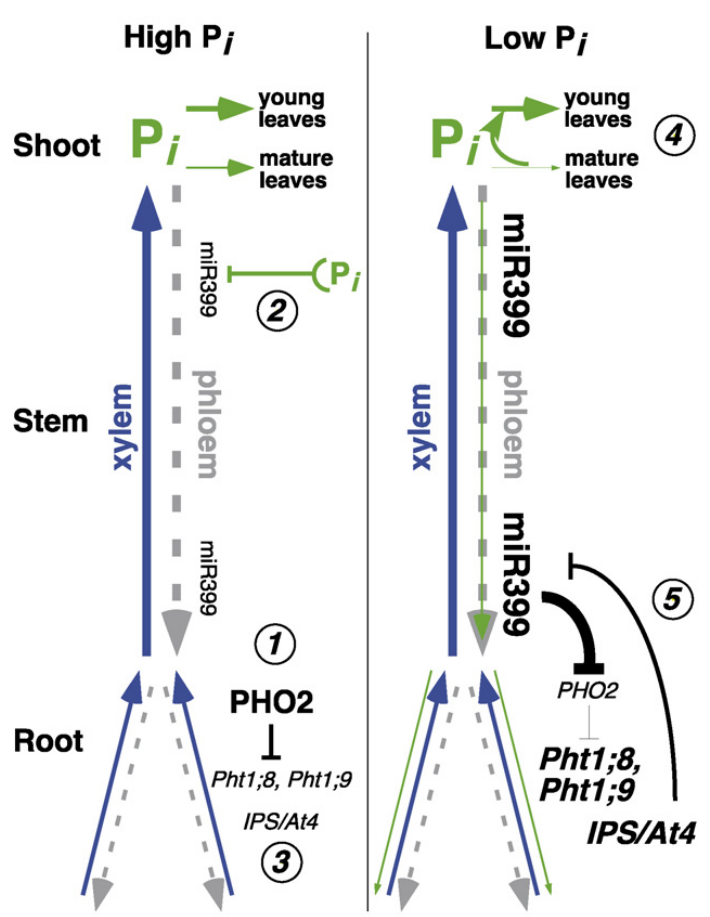

Figure 1. Suggested three component system responsible for the regulation of P_i homeostasis. (8)
According to this proposed signalling module PHO2, miR399 and IPS/At4 genes is responsible for the regulation PHO2 in the roots and controls P_i allocation to the shoots. **[1]** high PHO2 activity under adequate P_i supply, **[2]** miR399 expression is low under High P_i conditions, **[3]** IPS/At4 expression is low under High P_i conditions, **[4]** under P_i limitation miR399 expression is strongly activated, **[5]** miR399 is transported to the roots and suppresses PHO2 expression.

It is suggested that a 3 component system that is comprised of a family of phloem-mobile microRNAs (*miR399a-f*), their target gene, an E2 ubiquitin conjugase-related enzyme (UBC24) or otherwise known as *PHO2* and a family of non-coding RNAs, the IPS/At4-like genes work together in a regulatory module to control P_i allocation and redistribution. This module was elucidated in a series of publications and shown to control the activity of *PHO2* in plant roots that in turn regulate the allocation of P_i to the shoots of the plant (9-15).

When the plant is exposed to adequate amounts of P_i *PHO2* is highly active and suppresses the expression of the high affinity P_i transporters Pht1;8 and Pht1;9. Under high P_i conditions miR399 and

IPS/At4 expression are also low, but IPS/At4 expression is further down-regulated by *PHO2* under high P_i concentrations, keeping the regulatory module highly responsive to changes in P_i concentration.

When P_i supply is high, P_i is transported to both young and mature leaves. P_i limitation in turn strongly induces the expression of miR399, particularly in the shoot tissues. The mature miR399 transcripts are then transported via the phloem tissues to the root system where they interact and bind to the 5'-UTR of *PHO2* transcripts leading to degradation of the mRNA. The mRNA degradation of *PHO2* in turn leads to lower *PHO2* protein levels. Lower *PHO2* levels results in a lower inhibition of the expression of Pht1;8 and Pht1;9 and thus higher P_i uptake rates and higher P_i translocation to the shoots are induced.

When P_i levels are low, P_i is translocated from mature leaves to the younger leaves, and in order to maintain root meristem growth for the longest possible duration, P_i is also resupplied to the roots. As the low P_i response progresses, the inhibitory effect of *PHO2* on IPS/At4 wears off and expression is induced. This results in a form of feedback inhibition called target mimicry (15) where IPS/At4 acts as a ribo-regulator by inhibiting the silencing effect of miR399 on *PHO2* mRNA, fine tuning the protein levels to allow the plant to respond more rapidly to P_i supply and demand.

Interestingly enough all the members of this regulatory module are also expressed in the plant shoots although no direct function in these tissues has been identified yet. A long path still remains ahead of us before we can start to use our knowledge of these regulatory networks to improve crop nutrient efficiency. Several questions remain unanswered, mainly concerning the mechanisms that act upstream and downstream of this regulatory module. Which mechanism is utilised to perceive P_i concentrations and how many such mechanisms exist? Which mechanism of P_i detection is utilised in the upstream activation of miR399 and IPS/AT4 gene expression? What are the substrates of *PHO2* and how do these substrates mediate the over-expression of the high affinity P_i transporters Pht1;8 and Pht1;9? We also do not know the mechanism of transport of the miR399 complexes via the phloem.

These are just the initial questions and several additional questions would probably need to be addressed along the way in order to answer these. One of the important questions relates to the role of the regulatory module in plant shoots and the P_i transporters involved here. We would also probably need to look at P_i flux at a cellular level in the source and sink tissues in order to fully unravel and understand the mechanisms behind the balance of supply and demand and how it is achieved.

Investigating P_i transport and division between the various intracellular pools were therefore the main aim of this study. The role of one of the high affinity P_i transporters in P_i homeostasis during photosynthesis was investigated. We also attempted to perturb the P_i concentration in the vacuole and investigated a new promising technology as a potential tool to study the effect of these perturbations. The following sections provide an overview of the various aspects of this work to help the reader understand the theory behind the technology and choice of experiments.

Phosphate transport

P_i required by the plant needs to be taken up from the soil and redistributed throughout the plant's various organs. The low availability of P_i in natural ecosystems has resulted in the ability of plants to tightly regulate the uptake and movement of P_i between various cells, organs and sub-cellular compartments (16). Regulation of uptake and redistribution is largely controlled through a complex network of transport systems in the plant membranes. Substantial progress in the clarification of the details of these transport mechanisms was made in recent years through the adoption of molecular techniques and the completion of the sequencing of the *Arabidopsis* genome. Together, physiological and molecular evidence is starting to build a strong case for the fact that healthy plant growth and development largely depend on the plant's ability to coordinate a large number of P_i transport systems in various parts of the plant (16) and each family of transporters have a number of members. Table 1 summarises all the phosphate transporter families identified in *Arabidopsis*. Several reviews exist detailing the various transporters and their involvement in the overall process and can be consulted for thorough explanations about this vast field (4, 6, 16-19). Here it is only tried to summarise what is currently known about the various transporter families and their role in P_i transport and metabolism.

Table 1 *Phosphate transporters of* Arabidopsis thaliana

Gene name	***Gene ID***	***Suggested Function***
PHT1, 1	At5g43350	H^+/Pi symporter
PHT1, 2	At5g43370	H^+/Pi symporter
PHT1, 3	At5g43360	H^+/Pi symporter
PHT1, 4	At2g38940	H^+/Pi symporter
PHT1, 5	At2g32830	H^+/Pi symporter
PHT1, 6	At5g43340	H^+/Pi symporter
PHT1, 7	At3g54700	H^+/Pi symporter
PHT1, 8	At1g20860	H^+/Pi symporter
PHT1, 9	At1g76430	H^+/Pi symporter
PHT2, 1	At3g26570	Chloroplast H^+/Pi symporter
PHT3, 1	At5g14040	mitochondrial Pi translocator
PHT3, 2	At3g48850	mitochondrial Pi translocator
PHT3, 3	At2g17270	mitochondrial Pi translocator
AtTPT	At5g46110	Triose phosphate/Pi translocator
AtPPT	At5g33320	phosphoenolpyruvate/Pi translocator
AtGPT1	At5g54800	glucose 6-phosphate/Pi translocator
AtGPT2	At1g61800	glucose 6-phosphate/Pi translocator
AtXPT	At5g17640	Pentose phosphate/Pi translocator
PHT4;1	At2g29650	Plastid thylakoid membrane Pi transporter
PHT4;2	At2g38060	Plastid Pi transporter
PHT4;3	At3g46980	Plastid Pi transporter
PHT4;4	At4g00370	Plastid Pi transporter
PHT4;5	At5g20380	Plastid Pi transporter
PHT4;6	At5g44370	Pi transporter in Golgi membrane

Several P_i transporters were identified in plants involved in the movement of P_i into the plant and the redistribution of the available P_i between the various plant organs and compartments in the cell (Table 1). Based on sequence homology these transporters were classified into several families: PHT1 (plasma membrane), PHT2 (plastid inner envelope), PHT3 (mitochondrial inner membrane), PHT4 (plastid

thylakoid membrane and Golgi membrane) and pPT (plastid inner envelope) (3, 20, 21). Despite the large number of P_i transport proteins identified to date, evidence exists to support the presence of several other transport activities in plants that still need to be discovered. Unexplained activities identified include, export of P_i from amyloplasts not coupled to the transport of phosphorylated organic compounds (22), import of P_i into the vacuole (23) and the export of P_i from the Golgi, where it is generated as a by-product of glycosylation reactions (24). Several discoveries therefore still need to be made before P_i transport and the process of P_i homeostasis can be fully understood.

Cellular P_i homeostasis is strongly linked to the ability of the plant to tightly control the concentration of P_i in the various sub-cellular compartments (6). The vacuole is the major compartment in the plant cell responsible for the storage of P_i (17). The exchange of P_i between the cytoplasm and the vacuole is however relatively slow (25, 26) and is probably not sufficient to meet the short term cytoplasmic P_i demand. To demonstrate this Loughman *et al.* (1989) fed plants with P_i sequestering agents like mannose, this rapidly depleted the available P_i in the cytoplasm, although an abundant supply of P_i was present in the vacuole (27). This results in the so called P_i limitation of photosynthesis that occurs when photosynthesis is limited by a low P_i supply, even though sufficient P_i is present in the vacuole but not metabolically available in the short term (28, 29).

Over longer periods of time it is however critical that P_i concentrations are well controlled during photosynthesis. Until very recently it was believed that during P_i deficiency P_i concentrations in the cytoplasm is at around 0.2mM (5mM or more under normal conditions) (3). In a study by Pratt and co-workers (2009) it was, however, shown that the P_i concentration in the cytoplasm is around 60-80 µM and quickly drops to below 15 µM under P_i limitation (7). The concentration of P_i in the plastids and cytosol directly influences photosynthesis and carbon partitioning during the light-dark cycle (30). The importance of P_i in carbon partitioning was confirmed with experiments performed with isolated chloroplasts (31, 32). Drastic changes in P_i concentrations in the cytoplasm and chloroplasts influences photosynthetic activity and carbohydrate partitioning and therefore needs to be finely balanced for metabolism to perform optimally (28, 33). These processes are therefore dependent on the transport of P_i across the chloroplast membrane. Any imbalances could result in serious problems affecting photosynthesis and other cellular processes.

Nearly all P_i transport investigated in the chloroplasts was attributed to members of the plastidic phosphate translocator (pPT) family (21). These transporters are responsible for the exchange of P_i for phosphorylated C3, C5 and C6 compounds with a passive counter exchange mechanism and can be found on the inner envelope membrane of plastids. This counter exchange is strictly coupled and the strict stoichiometry is important because it ensures that each phosphoester exported is replaced by a P_i molecule maintaining the metabolic balance. This family of transporters were first identified with the triose phosphate/P_i translocator (TPT) that is involved in photosynthesis, where it exchanges P_i for the

triose phosphate end products of photosynthetic activity (34). This transporter is almost exclusively expressed in photosynthetic tissues. The TPT route of P_i transport is then also the major pathway whereby carbon is allocated to the cytosol during the day and acts as the primary route for P_i import into chloroplasts. Conversely, the rest of the members of the pPT family of transporters are involved in the export of P_i from the plastid in exchange for metabolic intermediates from the cytosol needed for biosynthetic processes in the plastid. These transporters include the glucose 6-phosphate/P_i translocator (GPT), xylulose 5-phosphate/P_i translocator (XPT) and phosphoenolpyruvate/P_i translocator (PPT) (35-37). Expression of XPT and PPT was found in both heterotrophic and photosynthetic tissues, whereas GPT is expressed only in heterotrophic tissues (38).

Times of P_i limitation represent a completely different scenario where the cell needs to remobilise P_i stored in various compartments and in P_i stores throughout the plant. During such times P_i is recycled by degrading intermediates, structural macromolecules and cofactors (39). Various physiological responses are observed in P_i stressed plants including an increase in root/shoot ratio (40), changes in the architecture of the roots (41, 42), preferential root production in soil patches containing higher P_i concentrations (43) and increased root hair length (44). The expression of high-affinity P_i transporters is upregulated under P_i limiting conditions in order to increase phosphate uptake (45, 16). Another important mechanism utilised is the recycling of stored phosphate from storage compartments and other pools (39). This coincides with an increase in the expression of RNAses (46) and phosphatases (47, 48) as well as an increase in organic acid secretion (49).

Remobilisation of P_i from high P compartments such as the vacuole and plastids would logically coincide with the upregulation of transporters in the membranes of these organelles. The mechanisms involved in this remobilisation process are however not yet fully elucidated. Several putative P_i transporter proteins have been shown to be up-regulated in periods of P_i limitation (17, 3, 39, 50). Uptake of P_i from the soil is mainly accomplished via the PHT1 family of P_i transporters, after which it is primarily transported symplastically to the xylem parenchyma for long distance transport to the above ground plant organs. The transport from parenchyma cells to the xylem is again mediated by another transporter-like protein (51, 30). When plants are supplied with sufficient amounts of P_i, most of it is transported via the xylem to the growing leaves that act as the main sink for P_i in the plant. The situation however changes when the P_i supply becomes limiting, and P_i needs to be re-translocated from the older leaves to the younger leaves and the roots (52). Several investigations in *Arabidopsis* (53, 45), potato (54) and tomato (55, 56) have pointed toward the involvement of members of the PHT1 family of transporters in the translocation of P_i throughout the plant, rather than only being involved in the uptake of P_i from the soil.

In this study the role of the PHT1;5 transporter in supplying P_i to the plastid under P_i limiting conditions was investigated. This transporter was previously shown to be expressed in plant leaves and was therefore thought to be a good choice to be investigated for this role.

What is Poly phosphate and why is it important

Inorganic polyphosphate (PolyP) is an unbranched polymer made up of a few to several hundred orthophosphate (P_i) residues that are linked together by phosphoanhydride bonds (figure 2). PolyP can be found in nearly all living organisms and is thought to be a prominent precursor in the evolution of life (57). It is found in extreme environments like sewage treatment plants, volcanic condensates and deep-oceanic steam vents. PolyP is truly a molecule with many different functions in living organisms and its function varies depending on the organism or even sub-cellular compartment it is found in (58). Its function varies from being an energy and P_i storage molecule to acting as a chelator of metal ions and acting as a buffer in the cell or a cellular capsule for bacteria. It is also involved in making bacteria competent for transformation and helping cells deal with all kinds of stress such as growth, development and deprivation. In addition it is used for the disposal of excess P_i in the environment.

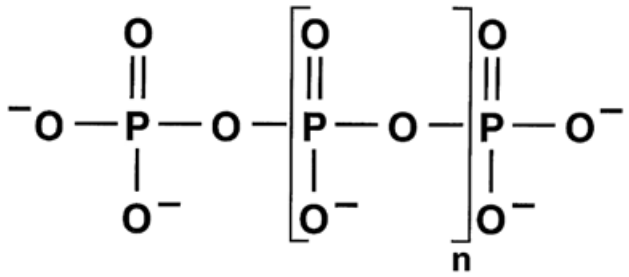

Figure 2. Structure of inorganic PolyP.

In 1888 L. Lieberman first discovered the presence of PolyP in yeast (59). It was however only much later in the 1940s and 1950s with the studies of Wiame (60), Ebel (61), Kornberg (62), Lohmann (63), and others that biochemists turned their attention to these compounds. PolyP was first discovered in bacteria as granules that stained pink with basic blue dyes (64). Upon first discovery, PolyP was wrongfully taken to be nucleic acids until it was viewed under the electron microscope and appeared to volatilise and was identified as PolyP (65).

Interestingly enough the function, synthesising enzymes and pathways responsible for its production seem to differ between the various sub-cellular compartments (66). This observation supports the notion that PolyP was one of the first molecules in living organisms and that it was involved in the origin of life and the beginning of evolution (67). The various aspects surrounding the function and distribution of PolyP and the regulatory and metabolic processes that it is involved in are thoroughly discussed in a recent review by Kulaev *et al*. (66).

Although PolyP was found in almost all living organisms studied to date, the levels found in microorganisms are far higher than those found in higher eucaryotes (57). In animal cells PolyP is found in the nucleus (68), but very little is known about the presence of PolyP in plant cells. The presence of PolyP is however widespread in lower plants (69), but apart from the transgenic introduction of PolyP into the chloroplasts of potato plants (70) no recent articles exist where the presence of PolyP was demonstrated in higher plants. The role and presence of PolyP (if any) in higher plants is therefore still unknown.

The properties of PolyP (like other polyanions) shifts the absorption of basic dyes bound to it into a higher wavelength and is one of the easiest ways to differentiate between PolyP and nucleic acids. PolyP was also historically used in the identification of medically important bacteria such as *Corynebacterium diphtheriae*. In the 1940s PolyP played a part in the major biochemical problem of how P_i is fixed by an anhydride bond to ADP in oxidative phosphorylation (72). *Escherichia coli* (*E. coli*) is an organism that has yielded many biological insights over the years and even though there is no visible PolyP content in this organism, it expresses an enzyme (poly P kinase [PPK]) that reversibly produces PolyP from ATP in the following manner (73, 74).

$$n\mathrm{ATP} \leftrightharpoons \mathrm{poly\,P}_n + n\mathrm{ADP}$$

Astoundingly, although the presence of PolyP was first discovered in yeast more than a hundred years ago, the synthesis pathway responsible for the production of PolyP in the yeast vacuole (the primary compartment in which PolyP is found in this organism) was only very recently demonstrated (75). Evidence for a pathway responsible for its production was provided by genomic expression analysis that was performed by Ogawa *et al.* in December 2000 (76). In the study by Ogawa, 21 genes were found to be involved in the PHO regulatory pathway of which 8 had no previous function assigned to them, the genes were designated PHM1 to PHM8. The PHM3 and PHM4 single mutants and the PHM1/PHM2 double mutant were all found to be severely limited in the accumulation of PolyP and P_i, while the phenotype of PHM5 suggested that this gene product was responsible for PolyP breakdown in yeast. The authors suggested that the PHM1 to 4 gene products forms a complex in the yeast tonoplast membrane that somehow synthesises PolyP while importing P_i into the vacuole. It was only 9 years later that Hothorn *et al.* then expressed the catalytic domains of the PHM 1, 2 and 3 proteins (Vtc2p, 3p and 4p) in *E. coli*. and showed compelling x-ray crystallographic evidence that the formed complex is responsible for PolyP production in yeast, detailing the first mechanism of PolyP production in eukaryotes.

In this study the genes responsible for the production of PolyP in the yeast vacuole were expressed in *Arabidopsis* in order to generate an additional P_i pool in the plant vacuole and in this way perturb the available P_i pool in this compartment. The idea was to investigate the plant's response to the

perturbed P_i concentration in the vacuole and hopefully learn more about the plant's homeostatic control mechanisms.

Förster / Fluorescence resonance energy transfer (FRET) as a tool to study intracellular metabolite concentrations

The introduction of fluorescence as a tool for biological measurements have revolutionised the field of microscopy and fluorometry as tools for biological measurements. For *in vivo* studies the use of small molecule dyes and genetically encoded fluorophores have set new horizons to what is possible with these techniques. New devices for spectral imaging, confocal technologies, pulsed lasers, quantum yield cameras, anisotropy decay imaging, polarisation microscopy and fluorescence lifetime imaging (FLIM) have provided many new approaches for the use of microscopic data in a quantitative manner (77). The combination of new types of dyes and fluorescent sensors with the availability of these tools has enabled the detection of changes in cellular processes as well as metabolite concentrations with sub-cellular resolution (78). Although the use of ratiometric fluorescent dyes had a major impact on this field of study, they are limited by their membrane permeability. The use of genetically expressed fluorophores on the other hand can be introduced into any cell that can be genetically manipulated. These sensors also have the advantage that they can effectively be targeted to defined sub-cellular compartments with the use of specific leader peptides or other targeting sequences on the genetic level (79, 80).

The simplest form of these sensors is the fluorescent proteins themselves, where the sensitivity of the protein for specific conditions are being utilised for the detection of intracellular changes, such as pH (81). Another, more advanced approach was the development of sensors exploiting the change in protein conformation upon binding. These sensors monitor protein conformation changes by means of FRET. FRET has been used for quite some time, the resonance energy transfer between the tryptophanes in a protein can for instance be used in structural analyses (82). In principle the sensors consists of a protein that changes conformation upon binding of a ligand. This protein is then fused to FRET reporters that can be detected and used to monitor the concentration of the ligand *in vivo*. By far the largest proportion of these sensors employ the use of GFP variants as FRET reporters, but recently the use of small molecule dyes have also been demonstrated (83, 84).

FRET can be defined as a 'long-range' dipole interaction between molecules through resonance dipole-dipole coupling occurring in the pico- to nanometer range (85) and is therefore at a scale similar to protein conformation changes. The principle of FRET is based on a quantum mechanical effect between a given pair of fluorophores. When the donor dipole is excited energy is transferred to the acceptor dipole (86, 87). In reality this means that part of the energy absorbed by the donor is transferred to the acceptor and emitted in the spectral emission wavelength of the acceptor. A very

thorough review by Okumoto *et al*. (2008) can be consulted for a more detailed explanation of FRET and the theory behind the principles of its use with fluorescent nanosensors (85).

The use of FRET in nanosensors is an extremely sensitive technique that is used for the detection of ligands binding to a specific protein. The conformational change of the protein is then related back to the concentration of the ligand that caused the change. The ratio of acceptor to the donor emissions change depending on the ligand concentration and can be used to quantify its concentration. FRET reporter systems typically consist of two fluorophores coupled with a polypeptide that binds a ligand with high specificity and a change in conformation caused by the binding is then detected via a change occurring in FRET efficiency. The first *in vivo* sensor that was based on the principle of expressing a fusion protein capable of detecting ligand changes by means of FRET was for the measurement of calcium concentrations (88, 89). It was developed based on the assumption that the large conformational change occurring when calcium binds to calmodulin would result in a change in the distance between the donor and acceptor pair that would in turn lead to a change in the emission ratio that could be related back to the calcium concentration.

An alternative construction of the sensor was based on the fusion of a calmodulin binding domain to calmodulin in order to enhance the allosteric interaction and the measured signal (90). Since then many more variations of the calcium sensor have been constructed (91). When these sensors were however evaluated by comparing their ability to function *in vivo*, it was found that some versions had superior efficiencies (92-94). The specific reasons for the difference in efficiency are however still elusive but needs to be addressed for the purpose of future sensor development.

Another sensor that was constructed is based on the sensitivity of certain versions of yellow fluorescent protein for halides and pH. This sensor consists of a fusion of enhanced YFP (eYFP) with enhanced CFP (eCFP) and can be used for the monitoring of chloride concentrations and was named 'clomelion' (95, 96). Several other FRET based sensors for signalling molecules have been developed over the last few years and thorough reviews on these are available for further information (78, 97-99).

These principles of sensor construction based on the binding of a ligand that are detected by FRET was employed in the construction of several metabolite sensors. In the case of these sensors the conformational change of periplasmic binding proteins (PBPs) from bacteria were utilised as basis for the construction. PBPs are a large family of binding proteins and include binding proteins for ions, carbohydrates, amino acids and peptides (100). The size of this family of proteins opens up the possibility of constructing sensors for literally thousands of molecules. Functional sensors that have been built using this principle are summarised in table 2.

Table 2. Functional fluorescence energy transfer (FRET) sensors available (85)

Class	*Molecule detected*	*Sensor name*	*References*
Pentose	Arabinose	FLIPara	(101)
	Ribose	FLIPrib	(102)
Hexose	Glucose	FLIPglu	(103, 104)
	Galactose	FLIPgal	(103, 104)
Disaccharide	Maltose	FLIPmal	(101)
	Sucrose	FLIPsuc	(105, 106)
Amino acid	Glutamate	FLIPe	(103, 107, 108)
	Arginine		(109)
	Tryptophan	FLIPW	(110)
Nucleobase	Purine	FLIPpur	(85)
Polyamine	Polyamines	FLIPpa	(85)
Ions	Calcium	Cameleon, TN-XL	(88, 111, 112)
	Phosphate	FLIPphos	(113)
	Protons	pHluorin, PTSilosarcus GFP	(81, 114)
	Halides	Clomelion	(95, 96)

In this study several affinity mutants of FLIPphos was evaluated for its applicability to multicellular plant organs in order to determine the usefulness of this sensor in the study of P_i homeostasis in higher plants. The purpose of this investigation was to utilise this technology in the study of the P_i perturbations generated in the other sections of this dissertation.

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-Chapter 3-

A high affinity phosphate transporter on the plastid inner envelope is essential for photosynthetic ATP production in phosphate-deprived environments[1]

Phosphorous in the form of orthophosphate (P_i) is an essential macronutrient involved in many essential cellular processes and amongst others required by plants in sufficient amounts to keep photosynthesis functioning at an optimal rate[1-3]. The concentration of available P_i in soil is extremely low, therefore all plants in their natural environment are always under some degree of P_i deprivation[4,5]. In periods of P_i limitation the P_i concentration in the cytoplasm drops into the low µM range[6,7]. All plastidial P_i transporters characterised to date, however, have a low affinity for P_i and would therefore not function under P_i limiting conditions[2,6,8,9]. Here we show that PHT1;5 is the plastidial transporter responsible for P_i uptake into chloroplasts under P_i-limiting conditions and that starch synthesis and degradation provides the carbon backbones for export from the plastids, which is contrasting the situation in well-nourished plants where P_i is transported in exchange for sugar-phosphates. It is generally accepted that P_i is transported across the chloroplast inner membrane by triose-phosphate/phosphate translocators (TPT), exchanging P_i for phosphorylated carbon intermediates[6]. The low affinity of these transporters for P_i would, however, not allow them to function in soil grown conditions where phosphate is limiting, which is mostly the case in natural ecosystems. We show that PHT1;5 is localised in plastids and responsible for P_i uptake under these conditions. Our results show that starch synthesis and degradation is much more important for carbohydrate partitioning in plants than previously thought and uncover the transporter utilised for P_i import into chloroplasts. Without this transporter photosynthesis stops functioning and plants cannot survive in their natural environment. P_i limitation is a major agricultural problem, normally solved by fertilisation, with sometimes devastating environmental effects. These findings reveal that the major pathway believed to be responsible for P_i uptake and carbon exchange in chloroplasts, only functions when plants are supplied with sufficient P_i and that another pathway is driving these processes under P_i limitation. Understanding this pathway is an important milestone in understanding how plants are functioning under more sustainable cultivation practises.

ATP is synthesised from ADP and P_i and is energised by the H^+ gradient generated during photosynthetic electron transport (supplemental Figure 1). This process in chloroplasts is dependent on the import of P_i but known plastidial P_i transporters cannot function at the predicted cytosolic P_i concentrations[7] under P_i-limiting conditions. Several putative P_i transporter proteins were shown to be up-regulated in periods of P_i limitation[10,6,11,12]. The PHT1 group of phosphate transporters[13] was identified by sequence similarity to the high affinity PHT1;1 transporter, expressed in the plasma membrane of root cells. Sequence analysis of this family also predicts similar characteristics for the rest of the transporters. When the various promoters of the PHT1 family of transporters were fused to reporter genes, the expression patterns of some of these transporters suggested previously unexpected functions[14]. In particular, PHT1;5 expression was strongest in the cotyledons of young seedlings as well as in senescing mature leaves, with reporter activity being the strongest in the vascular bundles and the phloem in particular. These results was interpreted to suggest that PHT1;5 plays a role in P_i remobilisation from older to developing tissues or to storage organs. These expression patterns were, however, obtained under conditions of sufficient P_i nutrition.

[1] This chapter was prepared for submission as a Letter to Nature and was written in the recommended style and format for this Journal. Additional experimental results are however still needed before final manuscript will be submitted.

When the expression pattern of this gene was investigated under P_i limiting conditions, it was found that it is highly induced in plant leaves under these conditions (Figure 1 (I)). In order to determine the sub-cellular localisation of this transporter *Arabidopsis* plants were transformed with a construct leading to the expression of a C-terminal GFP fusion protein with the transporter (Figure 2 (III)). Transformed seedlings were investigated using fluorescent microscopy and the PHT1;5-GFP fusion protein was found to be targeted to plastids (Figure 2 (I)). The localisation of this protein in the plastids suggests that P_i might be imported into chloroplasts through this high-affinity phosphate transporter rather than through TPT under P_i-limitation. In order to functionally analyze the role of PHT1;5 we obtained respective insertion mutants and generated transgenic lines with lowered expression of the gene (Figure 3 (I)). The plants mutated or down-regulated for PHT1;5 did not show any obvious phenotype or alteration in any of the metabolic or physiological parameters tested when they were grown under normal nutrition regimes. Changes only became evident when the plants were grown under P_i-limitation. To test the contribution of PHT1;5 towards P_i uptake into chloroplasts during periods of P_i limitation, chloroplasts were enriched and the total P content measured in P_i limited WT and PHT1;5 T-DNA insertion mutant (Figure 2 (II)) leaves as well as in the enriched chloroplast fractions. The P content of young leaves were 3 times higher in the wild-type plants than in the mutant plants and the chloroplasts fractions of the wild-type contained twice as much P as the chloroplasts derived from the mutant plants. These results suggests that PHT1;5 plays a major role in the uptake of P_i in chloroplasts during P_i limited growth conditions.

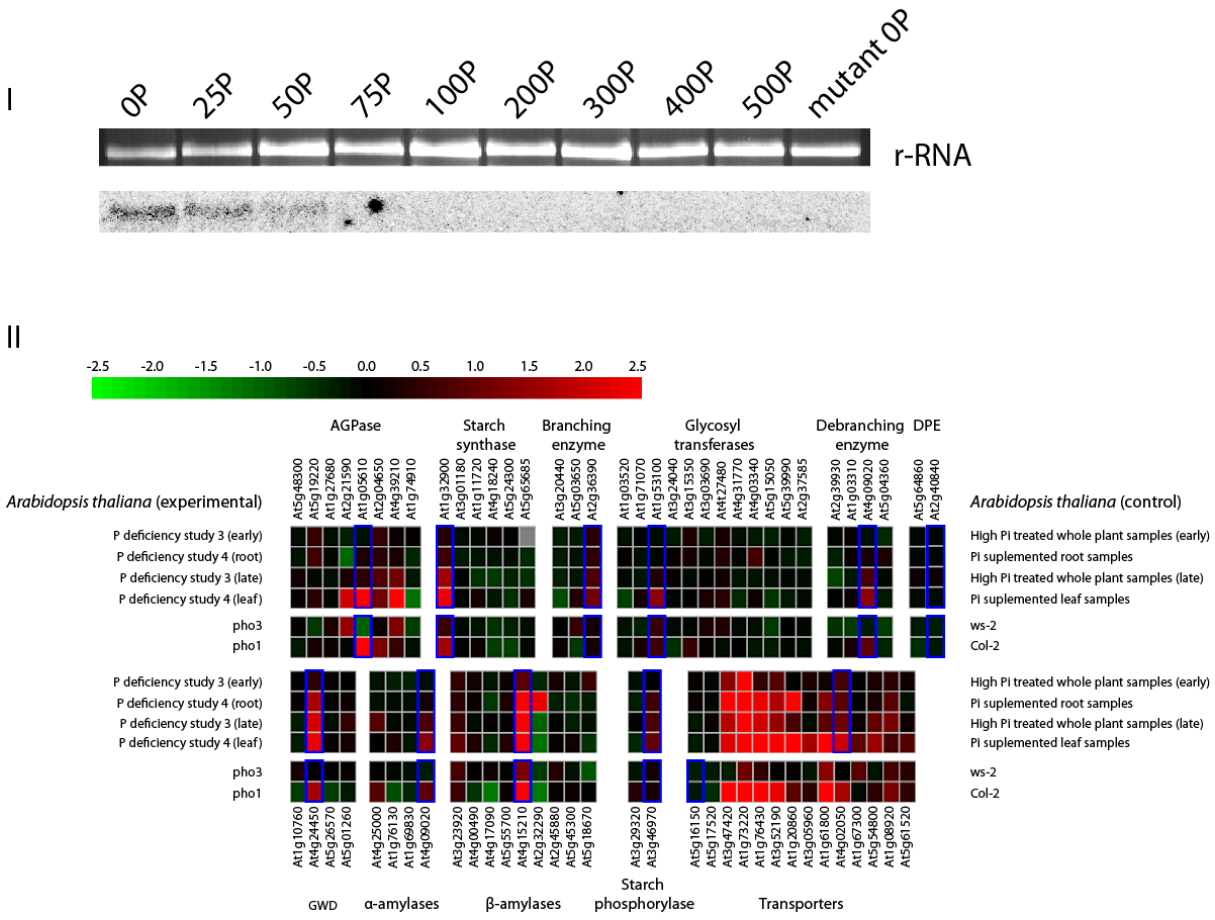

Figure 1. Expression analysis under P_i limiting conditions.

(I) Northern Blot showing the expression of PHT1;5 when plants are grown with varying concentrations of P_i units are in mg $P_i.L^{-1}$ nutrient solution. The PHT1;5 gene is only expressed when the plants are grown with a Pi concentration of 50mg.L^{-1} or lower. **(II)** Expression profile analysis of several genes involved in starch synthesis and degradation under P_i limitation. The legend on the left describes the experimental conditions and mutants used in the various expression profiles and the legend on the right is a description of the corresponding controls. A green colour indicates reduced levels of expression for the corresponding gene under the specific condition or mutation and a red colour indicates increased levels of expression. For specific intensities the colour index at the top of the figure can be consulted.

To test whether photosynthetic activity was indeed affected by the absence of the PHT1;5 transporter in periods of P_i depletion PSII efficiency (Φ_{PSII}), F_v/F_m, photochemical quenching (qP) and non-photochemical quenching (NPQ) were determined and compared as a measure of photochemical and non-photochemical photosynthetic potential of wild-type, mutant and antisense plants (Figure 3 (VI)). When photosynthesis was tested under conditions of sufficient P_i supply, no significant differences could be detected between wild-type, mutant and antisense plants (Figure 3 (III)). However, Φ_{PSII} and F_v/F_m were both significantly reduced in the P_i limited, mutant and antisense plants compared to P_i-limited wild-type plants. The decrease in the photosynthetic efficiency indicates a change in the pH gradient across the thylakoid membrane, inhibiting photosynthesis[15]. This change would most likely be the result of the reduced P_i content in the chloroplasts that does not allow for the regeneration of

ATP by ATPase, maintaining an increased ΔpH ((supplemental Figure 1) and Figure 2 (II)) across the thylakoid membrane. These conclusions are supported by the observation that ATP is not detectable in the plants with absent or lowered expression of PHT1;5 when grown under P_i-limitation, whereas unchanged ATP levels where found in all the different lines growing under sufficient P_i supply or the control lines under P_i-limitation (Figure 3 (IV). This finding supports our hypothesis that PHT1;5 is needed for the import of P_i into chloroplasts under P_i limitation to allow for photosynthetic ATP production. Plants lacking the protein will arrest ATP synthesis, and ultimately photosynthesis, consequently leading to death.

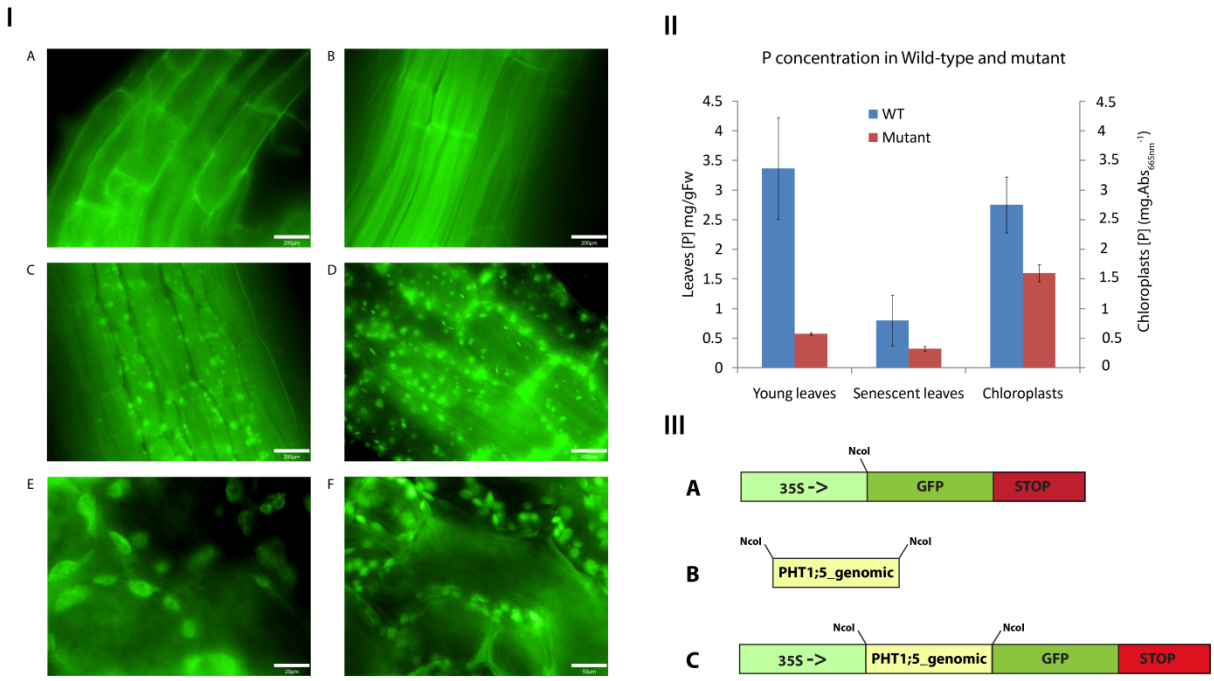

Figure 2. Sub-cellular localisation of PHT1;5 and effect on Pi distribution under P_i limitation.

(I) A C-terminal GFP fusion of PHT1;5 was detected in stably transformed *Arabidopsis* seedlings with untargeted GFP expressed as a control. **A** and **B** depicts the results obtained with GFP and **C** – **F** the targeting of the GFP fusion. **A** – **D** were taken with a free objective and E – F were imaged under 60 and 100x oil immersion objectives. Sizes of the respective scale bars are indicated below each. **(II)** Comparison of total P concentration of wild-type and mutant plants and chloroplasts from plants grown under P_i deprivation. Significant differences for both Young leaves and Chloroplasts (Students T-test) **(III)** Schematic representation of the expression vector used for the GFP fusion. (**A**) pCAMBIA1302 vector containing the GFP reporter gene (**B**) The PCR amplified genomic version of the PHM1;5 gene with built in restriction sites at either end (**C**) The completed GFP fusion construct in pCAMBIA containing the PHM1;5 gene in the correct orientation. Error bars represent standard deviation. Scale bar sizes are indicated below the scale bar on the figures in (**I**).

These results strongly indicate that a large proportion of the TPT activity is replaced by PHT1;5 during P_i depletion to allow for the import of P_i under limiting conditions. TPT is, however, also responsible for the export of phosphorylated carbon intermediates from the chloroplast to feed assimilated carbon into non-photosynthetic subcellular compartments or tissues. With this transporter not functioning,

another mechanism must exist to allow for the export of carbon intermediates from the chloroplast. In order to build a model on how carbohydrate might be exported from chloroplasts under P_i –limiting conditions, we made use of the *in silico* gene expression analysis tool Genevestigator[16] (https://www.genevestigator.com/gv/index.jsp) to screen for the effect of P_i limitation on the expression of genes involved in carbohydrate metabolism (Figure 1 (II)). 4 P_i limitation experiments were included together with the pho1 and pho3 mutants with lowered leaf P_i content. Interestingly 2 genes involved in starch degradation were highly up-regulated under these conditions, *i.e.* Glucan–water-dikinase-like (GWD2, AT4g24450) and β-amylase 5 (BAM5/ BMY1/ RAM1, At4g15210). BAM5 is, however, not a plastidial isoform, pointing towards the export of maltose from the plastid and the breakdown of cytosolic glycans playing a major role under these conditions, supporting the hypothesis of Lloyd and co-workers[17] (see supplemental Figure 1 for schematic representation). Upregulation of BAM5 could result in quicker glycan turnover that would support the increased carbon flux through this pathway during P_i limitation (supplemental Figure 1 **(6)**). Several genes involved in starch synthesis were also upregulated, such as At1g05610, encoding the minor small subunit isoform of ADP-glucose pyrophosphorylase (APS2) as well as a putative starch synthase gene (At1g32900).

The upregulation of these genes suggests that starch turnover during the light might be utilised by the plant as an alternative mechanism for generating carbon backbones which can be exported from the plastids as well as to release P_i from photosynthetic intermediates when P_i supply is limited. Glucose as well as maltose are well suited candidates for the export from plastids, as transporter molecules for both of them are present on plastid envelopes. It is noteworthy that the genes encoding the glucose and maltose transporters suggested for this role (At5g16150 and At5g17520, also included in Figure 1 (II)) are not affected in transcript levels under these conditions[18], which could mean that their expression levels under normal conditions are sufficient to account for all the required export rates.

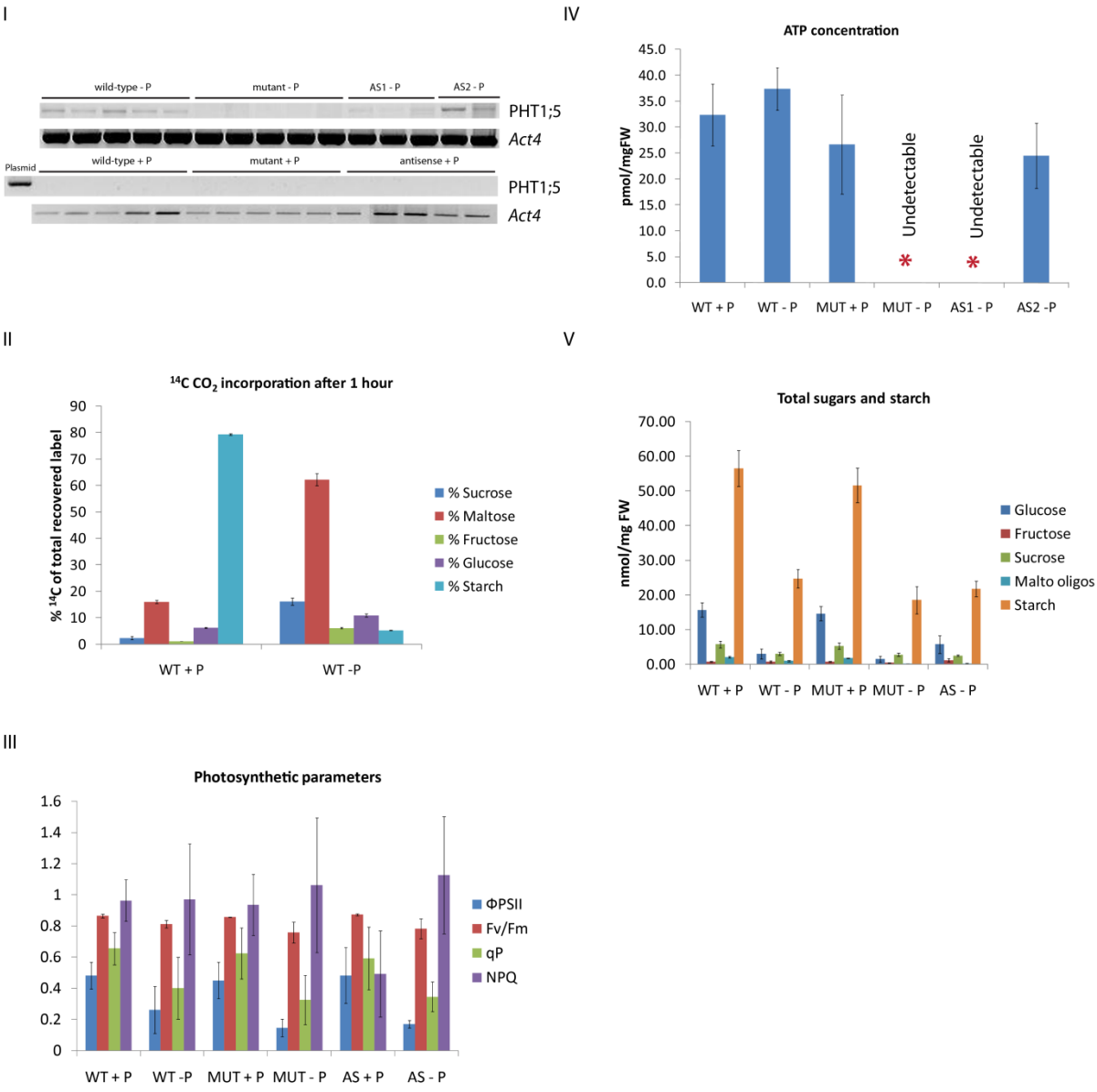

Figure 3. Metabolic and photosynthetic measurements.

(I) Verification of the expression of PHT1;5 with RT-PCR in plants used for metabolic analysis. **(II)** are ^{14}C CO_2 labelling results for plants labelled for 1 hour, results are expressed as percentage of label per total recovered label. **(III)** Photosynthetic parameters of wild-type, mutant and antisense lines. **(IV)** ATP concentration in wild-type, mutant and antisense plants. Antisense plants are divided into two groups with AS1 representing the plants with lowered expression and AS2 representing plants with similar expression than the wild-type **(V)** Concentration of total sugars in wild-type, mutant and antisense lines (values for MOS and starch are given in glucose equivalents). Error bars in all instances represent the standard deviation of measured parameters.

In order to test the hypothesis that P_i-limitation leads to increases in starch turnover, we fed P_i limited wild-type plants with ^{14}C labelled CO_2 for 1 hour and measured the amount of label allocated to glucose, fructose, sucrose, maltose and starch (Figure 3 (II)). The findings of the labelling experiment showed a decrease in the amount of carbon allocated to starch and massive increases of carbon diverted to maltose during P_i limitation. These results support our hypothesis that P_i depletion leads to

an increase in starch turnover to allow carbon partitioning to other compartments from the chloroplasts. We could not find any previous studies taking maltose into account during these types of experiments. Since the separation of maltose and sucrose was not optimal in our TLC separation, these experiments therefore need to be repeated in a setup where the separation is optimized. Unlabelled sugar and starch levels were also determined (Figure 3 (V)), all sugars and starch levels were lower under P_i depletion, but lower in the mutant plants under P_i limitation than the wild-type.

Some important aspects that would need urgent attention in future experiments would be to experimentally verify the K_m values for PHT1;5 for P_i and other phosphate intermediates. The importance of higher order malto-oligosacharides in the process of starch degradation should also be investigated in these mutants with ^{14}C labelling, using alternative separation techniques. We were unable to successfully separate sugars and higher order malto-oligosacharides with the TLC technique employed, but could identify significant amounts of label that did not migrate from the base of the TLC, this unidentified label is most likely incorporated into higher order malto-oligosacharides (See supplemental Figure 2). It might also be interesting to investigate the effect on the Calvin cycle intermediates in order to get possible clues towards the downstream mechanisms involved in starch turnover during P_i limitation.

Together these results clearly demonstrate the importance of this transporter in maintaining photosynthetic activity under natural plant growth conditions, which is usually under phosphate limitation.

Methods

Mutant and Antisense screening

The NASC European *Arabidopsis* stock centre website (http://arabidopsis.info) was searched for T-DNA insertion mutants of the AT2G32830 gene and the SALK_106359 (BO) insertion mutant was ordered from the website (stock number N606359)[19]. PCR verification of the presence of the T-DNA insertion was done as described on the Salk Institute Genomic Analysis Laboratory's website (http://signal.salk.edu) by making use of the LBa1 primer with sequence 5'-TGGTTCACGTAGTGGGCCATCG-3' and forward and reverse primers for the AT2G32830 gene with sequences; fw: 5'-GCTCATGGTACTATGTTCTCTCGGGTCTGG-3' and rev: 5'-GGTGGTTGTGCCGAGAAGGTGGAGACCGTG-3'. DNA extractions for PCR analyses was done by grinding up leaf tissue from each plant in liquid nitrogen and utilising the modified CTAB method described by White *et al.* (2008)[20], the aqueous phase after the first chloroform cleanup step was collected and genomic DNA was isolated with the wizard DNA cleanup kit (Promega). 1µl of cleaned up DNA was used per PCR reaction and all PCR reactions was performed with an annealing temperature of 55 ^{0}C with GO-Taq Taq polymerase (Promega), using buffers and recommendations of the manufacturer. *Arabidopsis* Col-0 was used as wild-type (WT) control line in all analyses.

Antisense plants were generated by cloning a 500 bp fragment of the PHT1;5 gene into pCAMBIA in the reverse orientation. The gene fragment was amplified from *Arabidopsis* cDNA with the following primers; fw: 5'-ATGAACGCAATCCACGAAGTCTTCA-3' and rev: 5'-TCAAACCGGGACTTTTCTACCGGAA-3'. The resulting DNA fragment

was cloned into pGEM-Teasy (promega) and subsequently cut out with *Bam*HI and *Eco*RI and cloned into pBLuescript SK$^-$. The resulting plasmid was again digested with *Xba*I and *Kpn*I and cloned into the same sites of the pBinAR-Kan[21] plasmid, from where the whole expression cassette was cut out and cloned into the pCAMBIA1300 plasmid with *Eco*RI and *Hind*III. *Agrobacterium tumefaciens* (strain GV3101) was transformed by using electroporation and *Arabidopsis* transformation was done by using the floral dip method[22]. Positive clones were selected for Hygromycin resistance on MS media containing 3% (w/v) sucrose and 50 mg.L^{-1} hygromycin and selected seedlings were used in further experiments.

Plant growth and maintenance

All soil grown plants were grown in the glasshouse in sunlight, with an average light intensity of 1000 µmol m^{-2}sec^{-1} and an average day length of 12 hours and temperature of 25 ˚C. Tissue culture grown plants were grown at a light intensity of 200 µmol m^{-2}sec^{-1} and a light cycle of 16 hours and a temperature of 25 ˚C on Murashige and Skoog (MS) media [23] with the addition of 3% (w/v) sucrose, 5mM MES and 0.8% (w/v) agar and pH adjusted to 5.7 with KOH.

P_i induction experiments

For selecting the appropriate P_i concentrations that would induce the expression of PHT1;5, plants were grown on vermiculite or palm peat and watered with ½ strength MS salts[23] at pH 6 (adjusted with KOH) as nutrient solution until 8 rosette leaves were visible. At this stage watering was commenced with ½ strength MS containing varying concentrations of KH_2PO_4, depending on the experiment, as follows; 0 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 75 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, 300 mg L^{-1}, 400 mg L^{-1} and 500 mg L^{-1}. This treatment was maintained for a period of 2 weeks. At this point plants started to flower and young plant leaves were harvested and frozen under liquid nitrogen and stored at -80˚C until further analysis. For further experiments the 0 mg L^{-1} and 400 mg L^{-1} treatments were selected as -P_i and +P_i treatments respectively, either inducing or not inducing the expression of the gene.

Nucleic acid extraction and analysis

RNA and DNA extractions were done with the technique developed by White *et al.*[20] and Northern blot analysis performed according to standard molecular techniques [24] and Venter *et al.*(2001)[25] respectively. First strand synthesis was done on isolated RNA with the SuperScript III First-Strand Synthesis System from Invitrogen (catalogue no. 18080051) by making use of Oligo(dT)20 primers, 1ul cDNA was used per 50ul PCR reaction. For Northern blots, probes were labelled with ^{32}P dCTP by incorporating the label into the probe with a PCR reaction, using the following primers; fw: 5'-ATGAACGCAATCCACGAAGTCTTCA-3' and rev: 5'-TCAAACCGGGACTTTTCTACCGGAA-3' using an anealing temperature of 55 ˚C and 35 cycles. RT-PCR analysis was performed using the following primers; fw: 5'-GCTCATGGTACTATGTTCTCTCGGGTCTGG-3' rev: 5'-GGTGGTTGTGCCGAGAAGGTGGAGACCGTG-3' at an annealing temperature of 55˚C and 35 cycles.

Chloroplast isolations

Chloroplasts were isolated from *Arabidopsis* leaves harvested from plants grown under P_i limiting conditions. 10 g Of plant leaves from each plant harvested after P_i induction as described in the P_i induction section (no P_i added to ½ strength MS) were used for the isolation of chloroplasts according to the technique described in Kubis *et al.*[26]. Isolated chloroplasts were washed 3 times to remove any P_i originating from other compartments and allowed to settle by gravitation and the supernatant aspirated with a glass pipette until all samples had a volume of 10ml. 100 µl aliquots were taken and extracted with 80% (v/v) acetone for chlorophyll determinations. For this, 400 µl 100% (v/v) acetone was

added to the 100ul aliquots and incubated at 60 ˚C for 30 min and the absorption of the extracts determined at 665nm in a Bio-Tek Instruments PowerWave X spectrophotometer (Bio-Tek, Winooski, VT). The absorption units were corrected for dilution and used directly to represent the relative amount of chloroplasts present in each extraction.

P content analysis

P content was determined by hydrolysing 1 – 2 g of fresh plant material or 5 ml of intact and washed chloroplasts in 10 ml hot H_2SO_4 by placing the leaf material or chloroplasts and H_2SO_4 in a glass tube and heating over an open flame while stirring, once the leaf material was dissolved completely, 30% (v/v) H_2O_2 were added, 100 µl at a time, while swirling the tube until the solution was completely clear. Bottles were allowed to cool and Pi concentrations determined. Analysis was performed on a Varian Liberty Radial ICP-AES. Calibration standards were prepared from a NIST traceable P standard in the same matrix as the samples, and the accuracy of the calibration verified with a quality control standard.

Construction and analysis of PHM1;5-GFP fusion

Sub cellular targeting of the PHT1;5 protein was determined by fusing the C-terminal end of the transporter protein to the N-terminal of the GFP in pCAMBIA 1302 (Figure 2 (III)). This was achieved by amplifying the at2g32830 gene from genomic DNA with gene specific primers containing built in *Nco*I restriction sites fw: 5'-ACAGAGAAAACCATGGCGAAAAAAGG-3' and rev: 5'-ACAAAATAACCATGGGAACCGGGACT-3' and cloning the resulting PCR fragment into pCAMBIA's *Nco*I site. Orientation was determined with restriction analyses. The resulting pCAMBIA plasmid, containing the fused transporter gene, together with the original pCAMBIA 1302 plasmid (as negative control), was transformed into *Agrobacterium tumefaciens* strain GV3101 with electroporation (Gene Pulser Xcell Electroporation System – Bio-Rad) according to the included protocols. Homozygous *Arabidopsis rdr6* lines[27] (gift from Scott Poethig, University of Pennsylvania) was transformed with the flower dip method[22] and transformed plants selected on MS media containing 50 mg L^{-1} Hygromycin, 3% (w/v) sucrose and 0.8% (w/v) agar, pH 5.7 (adjusted with KOH). Transformed plants were screened for highly fluorescent individuals, using a Leica MZ10 F epifluorescence stereo microscope. Fluorescent seedlings were analysed using an IX-81 Olympus microscope, equipped with a F-view-II cooled CCD camera (Soft Imaging Systems) coupled to an MT-20 (Soft Imaging Systems) xenon arc lamp.

Measuring of photosynthesis

The measurement of photosynthetic parameters were performed with a Hansatech instruments Fluorescence monitoring system (FMS2). F_v/F_m were first measured after dark adaptation of 2 hours with the provided leaf clips. After the determination of F_v/F_m plants were adapted to Actinic light at an 80% intensity, after the measured fluorescence intensity reached a baseline Φ_{PSII} was determined together with the other photochemical and non photochemical quenching parameters qP and NPQ respectively. All calculations were automatically performed by the instrument and data exported to Microsoft Excel for further analysis.

Metabolite determinations

For sugar and starch determinations plant leaf material was frozen in liquid nitrogen and 15 mg of frozen material was weighed off and extracted twice with 80% (v/v) ethanol containing 10mM MES at pH 5.9 and a third time with 50% (v/v) ethanol containing 10mM MES at pH 5.9. All supernatants were pooled and stored at -20 ˚C until analysis. Sugars and starch were determined as described by Cross *et al.* (2006)[28]. Malto-oligosacharides were determined in a similar way to the other sugars except for the addition of α-glucosidase prior to measurement. ATP extraction and quantification was performed as described by Bergmeyer (1985)[29].

^{14}C CO_2 labelling

CO_2 labelling experiments were performed as previously described by Walters *et al.* (2004)[18]. After P_i induction (as described above) plants were placed in a Perspex box and exposed to 100 µCi of labelled CO_2, generated by adding 3ml of formic acid to 500 µl of 1M $NaH^{14}CO_3$ (specific activity 0.2 Ci Mol^{-1}). Plants were placed in direct sunlight to allow them to photosynthesise for 15 min or 1 hour respectively. After labelling plants were removed in the fume hood and all above ground parts of individual plants placed in 10 ml hot (95 ˚C) 80% (v/v) ethanol to extract sugars. Extractions were performed 3 times for 30 min each and supernatants pooled and evaporated under filtered airflow. Leftover leaf material was dried at 80 ˚C and starch extracted by boiling for 1 hour in 1M KOH. Dried sugar extract were dissolved in 1 ml 80 % (v/v) Ethanol. The total amount of incorporated label was determined as previously described[30]. To determine the amount of label allocation to various pools, sugars were separated on TLC plates (HPTLC silica gel 60 F_{254}, MERCK) with Ethanol:pyridine:Acetic acid:H_2O (6:3:1:1) as the mobile phase[31]. 10 µg Of each glucose, fructose, maltose and sucrose were spotted as controls. For detection 10% (v/v) H_2SO_4 in Ethanol were sprayed onto the plates and it was baked at 110 ˚C until spots were visible. Parts of the TLC plate corresponding to the various sugar controls were scraped off, sugars redesolved in 80% ethanol and the amount of ^{14}C label determined as previously described[30]. Plates were also monitored on a phosphoimager scanner to detect migration of label[25].

***In silico* analysis**

For *in silico* analysis of transcript levels in response to P_i limitation the Genevestigator online analysis database were used (https://www.genevestigator.com/gv/index.jsp)[16].

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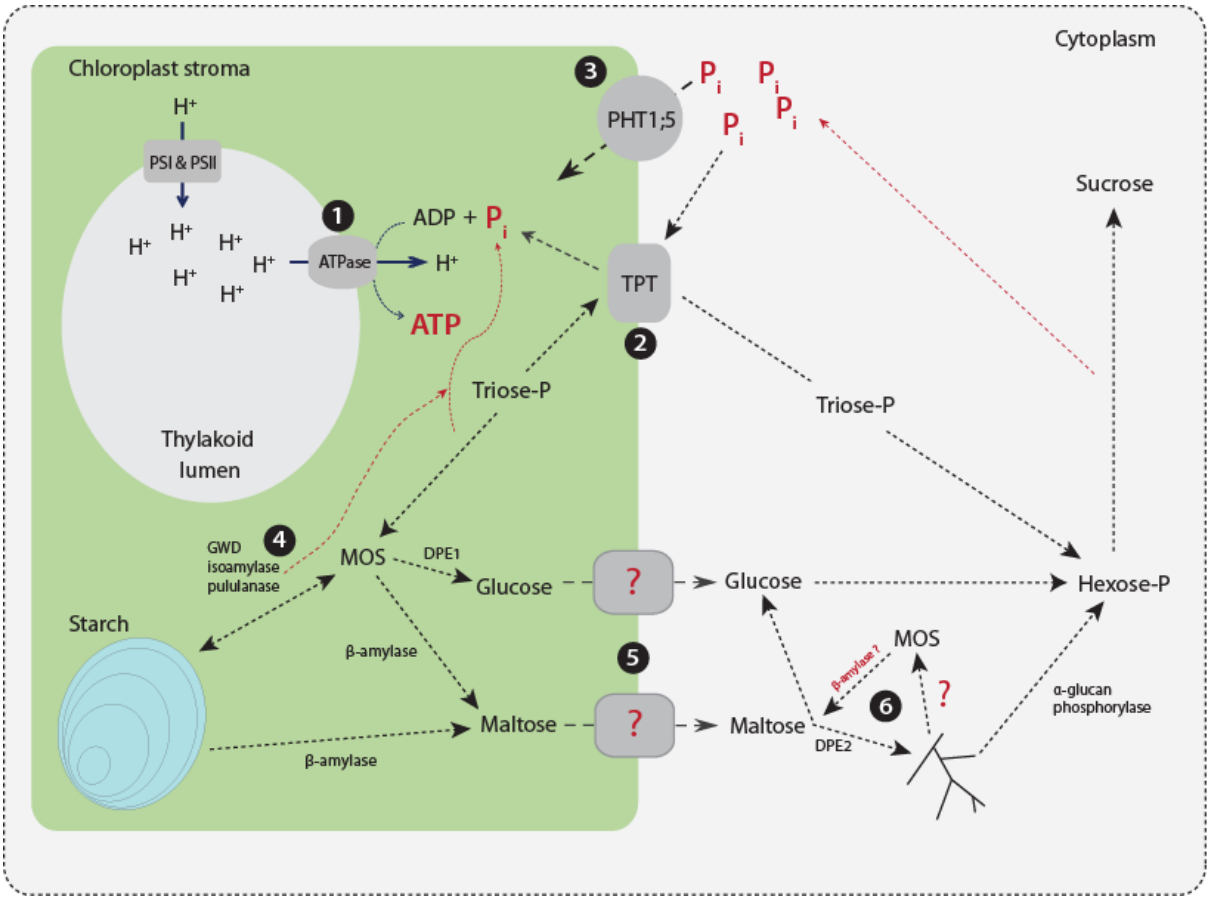
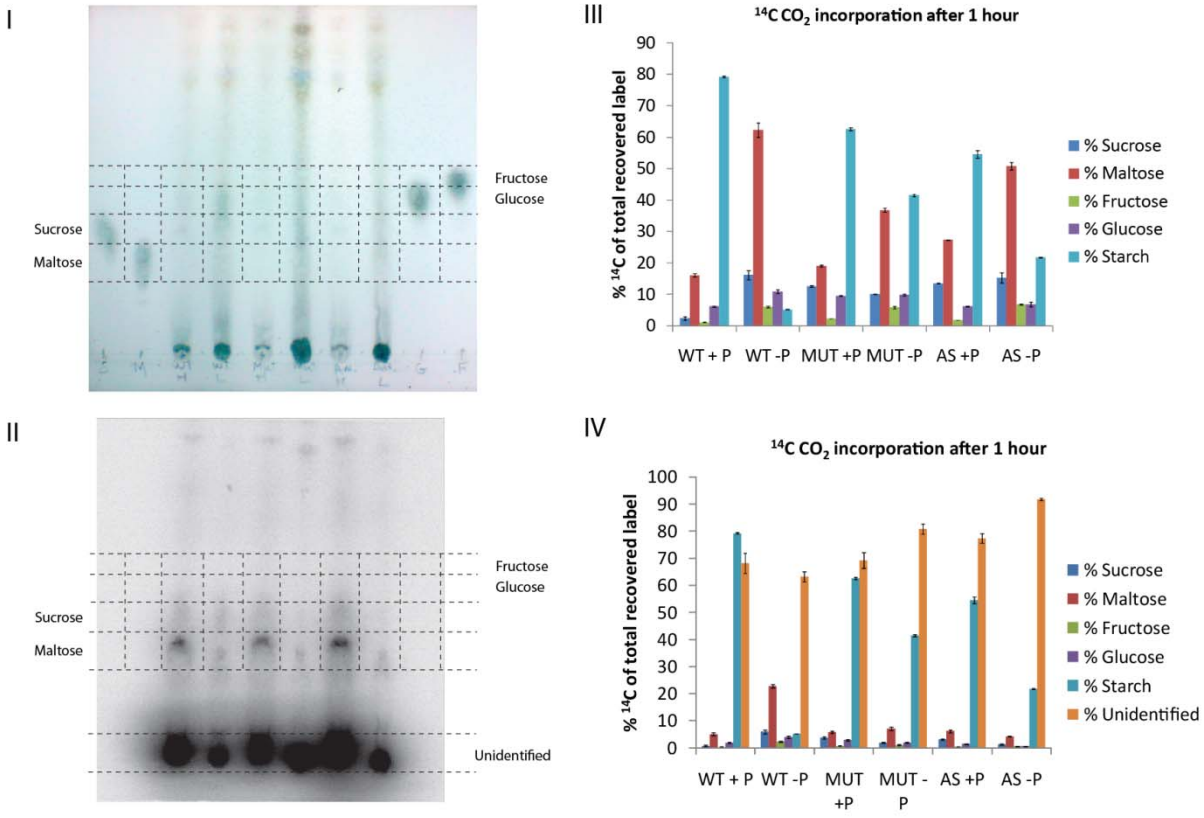
Supplemental material



Supplemental Figure 1. Representation of the role of PHT1;5 and carbon fixation during phosphate depletion.

(1) ATP synthesis is energised by the H^+ gradient generated by photosynthesis; P_i is required in optimum amounts for this process to continue. If P_i is limiting the H^+ gradient across the thylakoid membrane will increase to a point where photosynthesis will start to suffer. **(2)** During periods when adequate P_i is available to the plant it is exchanged for triose phosphate via the triose phosphate/phosphate translocator. This transporter, however, has a low affinity for P_i and are unable to function under periods of phosphate depletion and the import of P_i would have to be supplemented by other mechanisms. **(3)** The PHT1;5 transporter is located in the chloroplast membrane and is highly up-regulated during periods of P_i limitation. This transporter is suggested as the alternative for TPT during periods of P_i depletion, supplying the necessary P_i needed for photosynthesis. **(4)** Plants need to transport fixed carbon out of the chloroplast in order to supply metabolic processes with the necessary intermediates and to maintain cell metabolism at an optimal. Starch turnover can supply the cell with these intermediates and would release additional P_i through ATP hydrolysis to sustain the photosynthetic processes. **(5)** This would allow for the release of maltose and/or glucose from the chloroplast via as yet unidentified sugar transporters. **(6)** The process whereby maltose is converted into cytosolic glycans is still largely unknown, but a cytosolic β-amylase is upregulated during P_i limitation and it is suggested that this enzyme is involved with speeding up glycan turnover to hexose phosphate to allow for the extra carbon flux through this pathway during P_i limiting conditions.



Supplemental Figure 2. ^{14}C labelling determination.

(I) H_2SO_4 stained plate showing sugar standards. **(II)** Phosphoimager scan of plate, showing allocation of ^{14}C label with the grid indicating the position of the standards as it was cut from the plate for counting radioactivity. **(III)** Total amount of label allocated to various sugars with % of label allocated to each pool. **(IV)** same calculations as in **(III)** but with the unidentified pool taken into account.

-Chapter 4-

Expression of the polyphosphate polymerase complex from yeast in *Arabidopsis thaliana*.

Inorganic phosphate (P_i) is an essential macronutrient that is a component of nucleic acids and phospholipids and many other essential metabolites. In many organisms P_i is polymerized to form inorganic polyphosphate (PolyP).

PolyPs are linear polymers composed of a few to several hundred residues of orthophosphates that are linked together by energy-rich phosphoanhydride bonds. In 1888 L. Lieberman first discovered the presence of PolyP in yeast (1). It was, however, only much later in the 1940s and 1950s with the studies of Wiame (1947), Ebel (1948), Kornberg (1956) and Lohmann (1956), that biochemists turned their attention to these compounds (2-5). Since that time, PolyP has been found in almost all living organisms studied and localised to almost every sub-cellular compartment (6). The details of the function of PolyP in these organisms and compartments are, however, still largely unknown (7). Some biological functions that have been suggested include that PolyP acts as an energy store, a chelator of metal ions, a cellular pH buffer and that it may provide a channel for DNA entry into the cell (6).

The involvement of PolyP in the origin of life has been suggested by Harold in 1966, based on the energy properties of the molecule and its similarities to nucleic acids (8). Kulaev and co-workers wrote a review in 1999, summarising the function and metabolism of PolyP (9). Interestingly enough, they found that the function, synthesising enzymes and pathways responsible for PolyP production seem to differ between the various sub-cellular compartments (9).

PolyP is present in almost all living organisms studied to date, the levels found in microorganisms are far higher than those found in higher eukaryotes (10). Although PolyP is located in the nucleus in animal cells (11), a more widespread occurrence has been confirmed for lower plants (12). Apart from the production of PolyP in the chloroplasts of transgenic potato plants by expression of the polyphosphate kinase gene from *Escherichia coli* (13), no other reports where the presence of PolyP was conclusively demonstrated in higher plants exist.

Although the presence of PolyP was first discovered in yeast more than a hundred years ago, the biochemical pathway responsible for the production of PolyP in the yeast vacuole (the primary compartment in which PolyP is found in this organism) was only very recently demonstrated (14). Evidence for the pathway was provided by a genetic analysis (15). In this study, 21 genes were found to be involved in the PHO regulatory pathway of which eight had no previous function assigned to them. These genes were designated PHM1 to PHM8. In order to determine the function of the unknown genes, knockout mutants were generated for all of them. They showed that PHM3 and PHM4 single

mutants and the PHM1/PHM2 double mutant all had far lower levels of PolyP and P_i than the wild-type, while the phenotype of PHM5 suggested that this gene product was responsible for PolyP breakdown in yeast. The authors suggested that the PHM1 to 4 gene products form a complex in the yeast tonoplast membrane that somehow produces PolyP while importing P_i into the vacuole (15). These suggestions were confirmed when Hothorn and co-workers expressed the catalytic domains of the PHM 1, 2 and 3 proteins (Vtc2p, 3p and 4p) in *E. coli* and demonstrated with compelling x-ray crystallographic evidence the production of PolyP by this complex, suggesting a similar function in yeast (14).

These findings led to renewed interest in the question to whether this complex could be transferred to another organism in order to produce PolyP as an additional P_i pool in the vacuole of that organism. All eukaryotic proteins found in sub-cellular compartments, including the Golgi apparatus, endoplasmic reticulum (ER), vacuoles, lysosomes, plasma membrane and cell wall are sub-sorted from proteins originally targeted to the secretory pathway. The targeting of proteins to the secretory pathway is mediated by a hydrophobic signal sequence on the amino-terminal end. This signal peptide mediates the translocation of the specific protein to the lumen of the ER (16). At this point the signal sequence is spliced from the protein and some proteins undergo further post-translational modifications in the ER and Golgi network. As proteins are passing through the secretory pathway they undergo sorting to their respective targets, either by specific retention sequences in their primary sequence or by certain targeting information contained in the structure of the individual protein molecules (17). Once a protein enters the secretory pathway its default destination is secretion to the cell surface, unless some additional sorting determinants are present (17-20). Secondary sorting signals can include post-translational modifications or can depend on the primary, secondary or tertiary structure of the polypeptide (21).

Although no structural determinant or consensus sequence for targeting of yeast vacuolar proteins have been identified to date, some specific peptide sequences were demonstrated to be responsible for the sorting of individual proteins to the yeast vacuole (22-24). The fact that no common structural determinant or consensus signal peptide has been identified thus far signifies that a diverse set of factors are involved in the sorting process.

Some plant vacuolar proteins were shown to be correctly targeted to the yeast vacuole (25, 26). The signal sequences involved were isolated and are similar to the sequences identified in yeast and was confirmed to be sufficient to target other plant proteins to the yeast vacuole (27). These sequences were however not sufficient for the targeting of the same plant proteins to *Arabidopsis* vacuoles, indicating significant differences between the two systems (28). Subsequently it was demonstrated that the carboxyl-terminal domain of barley lectin is required for efficient sorting of this protein to tobacco vacuoles, indicating a higher degree of similarity between the two plant systems (29).

The introduction of PolyP in the vacuole of *Arabidopsis* as model organism would in theory provide an ideal mechanism of perturbing the P_i concentration in the cytosol of this plant. This would in turn give us the opportunity to study the effect on P_i homeostasis, that might lead to a better understanding of this process in plants. The minimal set of genes required for the production of PolyP in yeast vacuoles as proposed by Ogawa *et al.*, namely PHM2, PHM3 and PHM4, were therefore expressed in the higher plant *Arabidopsis thaliana*. While testing the possibility of transferring the PolyP polymerising activity from yeast to plants it also presented the opportunity to test whether these yeast membrane-bound proteins could be correctly targeted to the plant vacuole.

Our hypothesis was that expression of PHM2, PHM3 and PHM4 genes from *Sacharomyces cerevisiae* in *Arabidopsis thaliana* would be sufficient to transfer the PolyP polymerising activity, and that this would result in the accumulation of PolyP in the plant vacuole, perturbing the natural P_i concentration. We subsequently provide proof that the expression of these genes did indeed result in the accumulation of a detectable amount of PolyP in the plant *Arabidopsis thaliana*. This observation in itself provides additional proof that the PHM proteins are indeed responsible for the production of PolyP in yeast. The ability to transfer the PolyP polymerising activity from yeast to plants supports both the findings of Ogawa (2000) and Hothorn (2009) (14, 15). The low level of PolyP accumulation however did not provide a sufficient perturbation of P_i in this system and could therefore not be used for this purpose.

Materials and Methods

Plant material, growth and maintenance: The Col-0 ecotype of *Arabidopsis thaliana* was used throughout the study. Plants were grown under a 16 hour light, 8 hour dark cycle with a light intensity of 200 µmol $m^{-2}sec^{-1}$.

Molecular biology and transformations: All DNA manipulations were done according to standard protocols described by Sambrook *et al.*(30).

Construction of transgenic plants: The PHM2, PHM3 and PHM4 genes of *Sacharomyces cerevisiae* were amplified by PCR from yeast genomic DNA using the following primer sets;
PHM2 fw: 5'-CCGGGGTACCATGCTATTTGGTATTAAACTGGCTA-3' and rev: 5'-CCGGGTCGACTTATTCCCCAACCAAATTGAAGAT-3', PHH3 fw: 5'-GGCCGGTACCATGAAGTTTGGTGAGCACTTGAGCA-3' and rev: 5'-GGCCGTCGACTCATTTAGCAACTAGGTTGCAGAAA-3', PHM4 fw: 5'-GGCCATGGCTTCAGCACCATTATTACAAA-3' and rev: 5'-CCGGGGTTACCTCATAACTTAGTGTTAGCGTCATTG-3' at an annealing temperature of 55^{0}C for all, with KapaHiFi hi fidelity DNA polymerase (http://www.kapabiosystems.com), used at conditions recommended by the manufacturer. The PCR product for PHM2 had a size of 2500bp, PHM3 had a size of 2200bp and PHM4 a size of 400bp. PCR products were cloned into the PCR cloning vector pGEM T-easy (Promega) and sequence verified genes were excised and subloned into either the pCambia plant expression vector (http://www.cambia.org) or the pBinAR-Kan (31) vector. A pCambia plant expression vector was constructed by excising the 35S – Nos Promotor terminator cassette of pBinAR-Kan (31) with *Eco*RI and *Hin*dIII and cloning this into the pCambia 3300 vector. The constructed plasmid was designated pCam3300-35S. The PHM2 gene was then subcloned into pBinAR-Kan (containing the nptII resistance gene for kanamycin) and the PHM3 gene into pCam3300-35S (containing the Bar resistence gene for BASTA). The PHM4 gene

was cloned into pCambia-1301 (containing the hph resistence gene for hygromycin) with the *Nco*I and *Bst*EII enzymes, replacing the reporter GUS gene.

After construction of the expression vectors, plasmids were extracted and transformed into *Agrobacterium tumefaciens* strain GV3101 with electroporation (Gene Pulser Xcell Electroporation System – Bio-Rad) according to the included protocols. All *A. thaliana* transformations were performed according to standard protocols with the flower dip method (32) except that plants were co-transformed with all three genes. This was achieved by mixing all 3 *Agrobacterium* transformants in equal amounts (OD_{600} of 1 for each transformant) and using the mixture for plant transformation.

Plant selection and tissue culture: *Arabidopsis* seeds were harvested, surface sterilized with a 20% (v/v) bleach solution (Domestos brand) and washed three times with sterile distilled water. After sterilization the seeds were plated on solid MS media supplemented with 3% (w/v) sucrose, 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, pH 5.7 and the two antibiotics Kanamycin and Hygromycin, both at a concentration of 50mg.L^{-1} (selection media). After two weeks the surviving seedlings were taken off selection and planted in the growth chamber to harden off. After hardening off, further selection was performed by spaying the plants with the commercial herbicide BASTA (Bayer) at a concentration of 0.1% (v/v) in distilled water. Plants were sprayed repeatedly every two days for a period of two weeks or until all untransformed seedlings have died off.

After selection, leaves were harvested from the surviving plants for RNA extractions and RT-PCR analysis. Plants expressing all three genes were selected and seeds were allowed to set. For further analysis and callus initiation, seeds were harvested, surface sterilized and once again plated on selection media that was now supplemented with phosphinotrycin at a concentration of 50 mg.L^{-1} (in addition to the other antibiotics). Surviving seedlings were either planted out to harden off or placed onto callus initiation media. Callus was initiated by placing *Arabidopsis* seedlings on solid MS media (Sigma catalog number M5519) supplemented with 3% (w/v) sucrose, 5mM MOPS KOH (pH 5.7), 0.3% (w/v) phytagel, 0.5mM 2,4D and 0.05mM Kinetin in the dark. Callus that formed was transferred to new media every two weeks.

RT-PCR analysis: Total RNA was extracted with the method described in Venter *et.al.* (33). First strand synthesis was done with the SuperScript III First-Strand Synthesis System from Invitrogen (catalogue no. 18080051) and 1ul used per 50ul PCR reaction. PCR was performed with the same gene specific primers used in the amplification from yeast genomic DNA. PCR products were separated on 1% (w/v) Agarose gels, stained with Ethidium Bromide and visualized under ultra violet light.

Yeast growth and DNA extraction: *Sacharomyces cerivisiae* yeast was grown by streaking out on solid YPD media and growing overnight until single colonies were observed. Single colonies were used to inoculate 10ml overnight cultures that were in turn used for the inoculation of 200ml overnight cultures in liquid YPD media. All overnight cultures were grown at 30 ^{0}C for 16h. Genomic DNA extractions from yeast were done according to standard laboratory practices (30).

PolyP extraction: 200ml overnight yeast cultures were used as positive control for the extraction of PolyP. The yeast cells were pelleted at 6000 x g for 20 min and the pellet was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. Plant PolyP extractions were performed using wild type and transformed *Arabidopsis* plants grown until the onset of flowering, at which stage all above ground parts were ground with a mortar and pestle in liquid nitrogen. To extract PolyP, 10g of the finely ground samples were mixed with 10 ml of extraction buffer (300mM Tris, pH8, 100mM NaCl, 100mM EDTA, 2% (w/v) SDS and 5% (v/v) β-mercapto ethanol) and vortexed every 5 min for a total of 30 min at room temp. Extracts were then cleared by centrifugation at 10000 x g for 20 min. Supernatants were

extracted with equal volumes of chloroform and the upper phase was collected and stored at -20 ^{0}C until further analysis. Samples were concentrated by precipitation with 2 volumes of isopropanol and then washed twice with 70% (v/v) ethanol, dried and dissolved in 1 x TBE buffer. All samples were stored at -20 ^{0}C until further analysis.

NMR analysis: The ^{31}P spectrum of the analyte was obtained as previously described on a Varian 600MHz spectrometer utilizing a 10mm broadband probe after addition of 10 % (v/v) D_2O to the extracts (13). Commercial sodium phosphate glass type 65 (Sigma catalogue no. S6253) were added to the extracts at a concentration of 3 mg.ml^{-1} as PolyP control and methylenediphosphonate (MDP) at a concentration of 50mg.ml^{-1} were added to a capillary as reference. Compounds were identified by reference to MDP and chemical shifts were determined relative to the resonance of MDP set at 18.58 ppm and are quoted to 85% (v/v) orthophosphoric acid at 0 ppm.

PAGE analysis: PAGE analysis was done as previously described (15). 30 µl of PolyP extract was loaded onto the gel together with yeast extracts as positive controls. Extracts from wild-type plants were also loaded as negative controls. DNAse and RNAse treatments of the samples were done using the DNAse I (catalogue no. #EN0525) and RNAse A (catalogue no. #EN0531) enzymes from Fermentas according to the instructions of the manufacturer. PPX digestion of PolyP was done as described in Werner *et al.* (34).

Results and discussion

Higher plants are some of the few organisms that have not been shown to produce detectable levels of PolyP. The production of PolyP is, however, widespread amongst all other living organisms. Under the organisms producing PolyP is *Saccharomyces cerevisiae*, one of the best studied and well documented of all organisms. Although the enzymes responsible for the production of PolyP in *E. coli* have been well documented over the years, the mechanisms responsible for its production in yeast was still eluding scientists until very recently. It was only with the availability of powerful genetic tools and yeast knockout libraries that this discovery became possible, allowing Ogawa and co-authors (2000) to discover the genes essential for the production of this important polymer (15). After this discovery it again took another nine years before any further substantial advancements could be made in the understanding of the functioning of this enzyme complex with the crystallisation of the catalytic domain of the complex heterologously expressed and purified from *E. coli.* (14).

Although the results obtained by Hothorn *et al.* (2009) suggests that PolyP is synthesised by a complex of the protein products of PHM1, 2 and 3, mutation analysis done by Ogawa *et.al.* (2000) suggested that PHM3 and PHM4 together with either PHM1 or PHM2 were essential for the production of PolyP in the vacuoles of yeast (14, 15). We therefore set out to test the hypothesis that the expression of PHM2, PHM3 and PHM4 in plants would be sufficient for the production of PolyP in the plant vacuole by expressing these genes in *Arabidopsis thaliana*.

Arabidopsis transformation and selection

Transformed plants were selected with the respective selection agents and the four plants that survived the selection process were subjected to RT-PCR analysis and three of these expressed all three

transgenes (Figure 1A). The three lines were designated PolyP$_i$1, PolyP$_i$2 and PolyP$_i$4. After undergoing a second round of antibiotic selection and RT-PCR (Figure 1 B) it was shown that three lines were lacking PHM3 (Figure 1B PHM 3 lanes 9, 10 and 11) and that another two lines were lacking PHM4 (Figure 1 B PHM 4 lanes 4 and 5). The rest of the lines analysed were expressing all three genes on the mRNA level. One plant from each of these lines was selected for further analysis and designated PolyP$_i$1-2, PolyP$_i$2-2, and PolyP$_i$4 respectively. One of the lines, PolyP$_i$4, showed a very severe stunted and purple phenotype under selection and it was therefore not possible to harvest enough material for RNA extraction. However, under normal glasshouse conditions these plants showed no phenotype and callus initiation was normal. The other 2 lines were also phenotypically unaffected by the expression of the 3 genes.

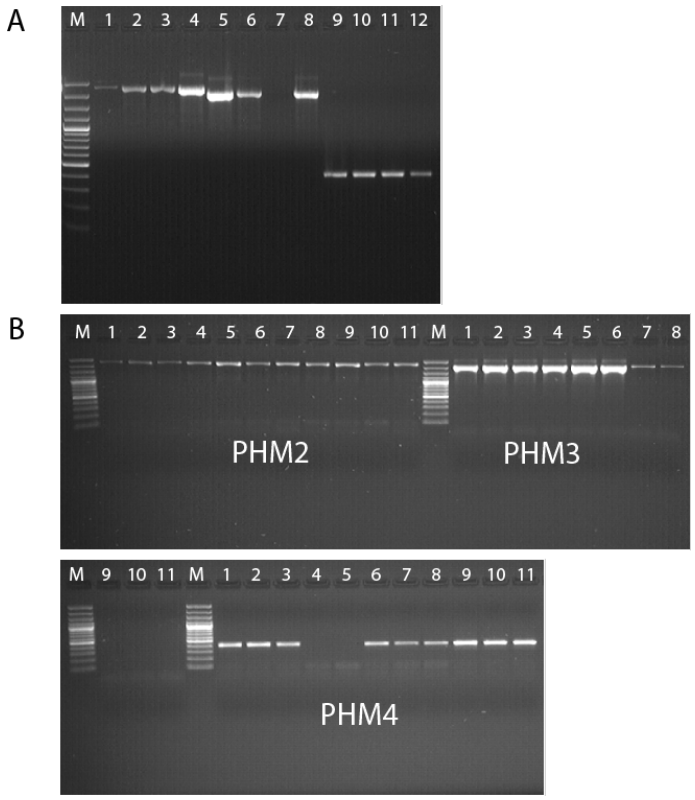


Figure 1. RT-PCR analysis of selected *Arabidopsis* plants transformed with PHM2, PHM3 and PHM4.

(**A**) Lanes 1 – 4 represent clones 1 – 4 analysed for the presence of PHM2; lanes 5 – 8 represent clones 1 – 4 analysed for the presence of PHM3; lanes 9 – 12 represent clones 1 – 4 analysed for the presence of PHM4. (**B**) Analysis of T_2 generation of plants before callus initiation and PolyP extraction. Lanes 1 – 6 represent extracts from clone 1; lanes 7 and 8 represent extracts form clone 2 and lanes 9 – 11 represent extracts from clone 3. The sizes of the amplified fragments were verified using molecular 1kb plus molecular marker from Fermentas (lane M).

PolyP extraction and analysis of transformed plants and callus.

Plant, callus and yeast cells were extracted with a highly buffered 2% (w/v) SDS solution containing high concentrations of EDTA to prevent the formation of complexes between the PolyP and cations in

the tissue. To determine if any detectable amounts of PolyP was present in the plant samples, extracts were separated with polyacrylamide gel electrophoresis (PAGE) and stained with Toluidine Blue, as previously described by Ogawa *et.al.* (15) (Figure 2). Plant callus extracts from PolyP$_i$2-2 and PolyP$_i$4 showed a clear purple smear on the PAGE gel, similar to that seen with the yeast extract, except that the smears were much lighter, signifying a far lower concentration (Figure 2). None of the plant leaf extracts however showed anything on the gel, even for the clones that did show a smear for the callus extracts.

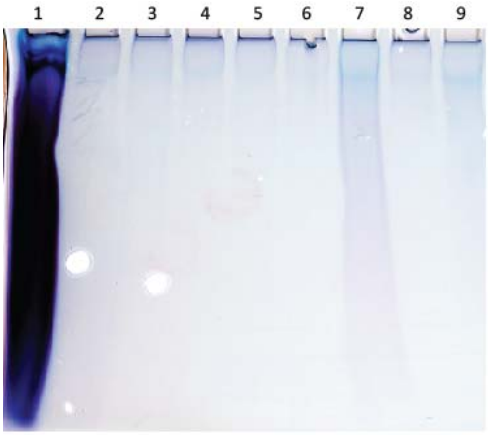


Figure 2. Polyacrylamide gel electrophoresis (PAGE) of plant extracts stained with Toluidine Blue.

Lane **1** - yeast extract, **2**- WT plant extract, **3** - WT callus extract, **4** - clone 1-2 plant extract, **5** - clone 1-2 callus extract, **6** - clone 2-2 plant extract, **7** - clone 2-2 callus extract, **8** - clone 4 plant extract, **9** - clone 4 callus extract.

In order to determine the identity of the purple smears on the PAGE gel and to attempt detection of PolyP in the whole plant extracts, D_2O was added to the extracts and the mixtures were subjected to ^{31}P NMR analysis. Commercial PolyP from Sigma (type 65) was included in the analysis at the concentration of 50mg.ml^{-1} as a positive control (Figure 3). The expected spectrum of PolyP was observed for the commercial PolyP in the extraction buffer and was used to identify the PolyP peak in the other extracts (**A**). In the spectrum observed for the yeast extract (**E**) the PolyP peak could also be clearly identified at 20.9 ppm. The same observation was also made for the wild-type *Arabidopsis* extracts to which the commercial PolyP was added. The spectra of **B** and **F** verifies that no peaks are observed in the 20.9 ppm range for either wild-type plants or callus. **D** was crucial in indicating the spectra obtained for ATP, of which the third peak is close to the PolyP peak of 20.9 and can be seen at 20.6. This observation was important in the differentiation between these two compounds in the spectra of the transformed plants.

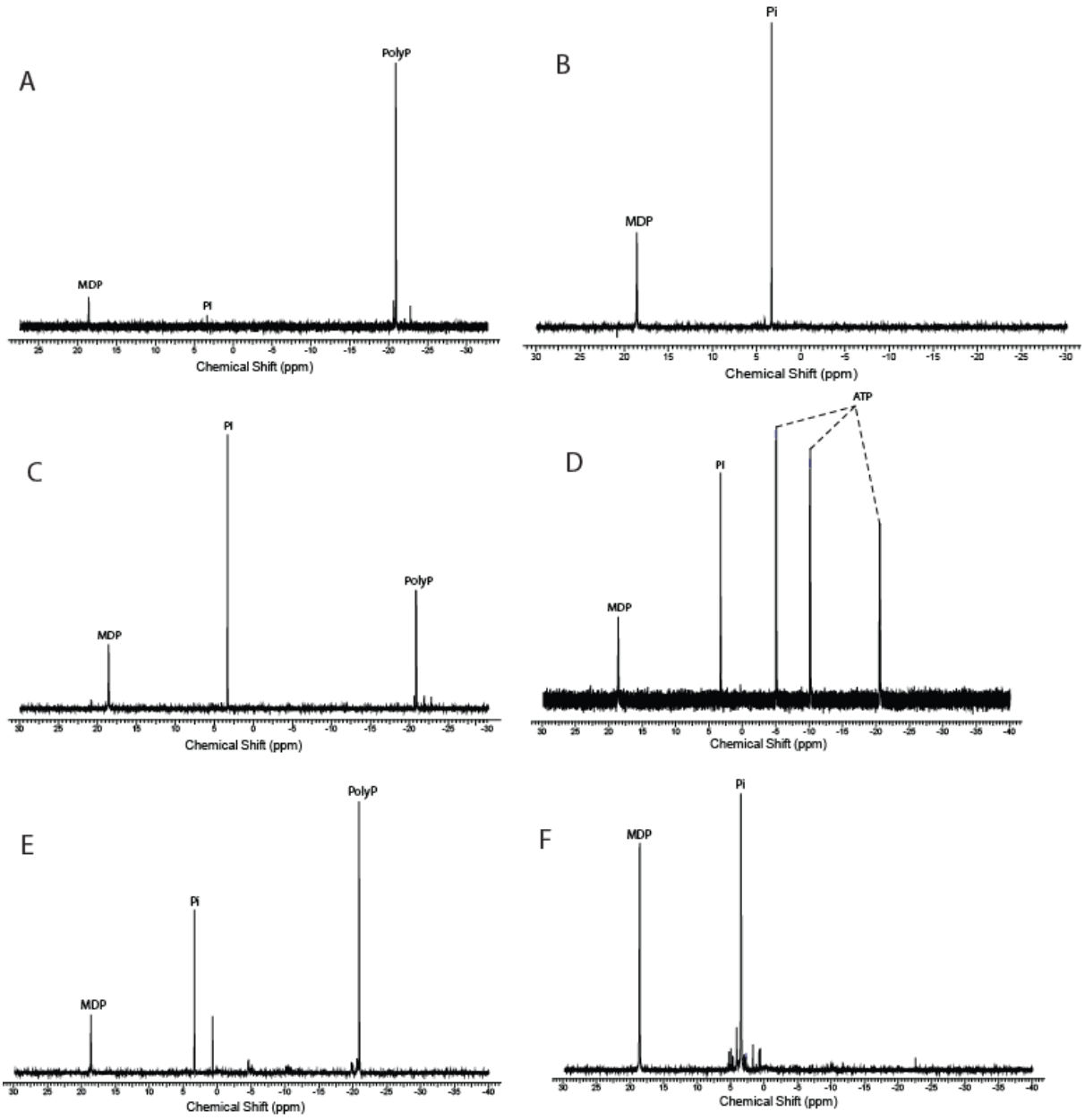


Figure 3. ^{31}P NMR spectra of control samples.

All spectra were referenced to MDP (methylene diphosphonate) as external reference in a capillary insert at a chemical shift of 18.58 ppm. **A**: ^{31}P NMR spectrum for the extraction buffer with added sodium phosphate glass type 65 (Sigma); **B**: wild-type *Arabidopsis* plant extracts; **C**: wild-type *Arabidopsis* extracts plus sodium phosphate glass type 65; **D**: wild-type *Arabidopsis* extracts supplemented with pure ATP; **E**: *Saccharomyces cerevisiae* extracts and **F**: concentrated wild-type *Arabidopsis* callus extracts.

No PolyP peak at 20.9 could be detected in any of the transformed plant samples using this method (Figure 4 A). To investigate the possibility that the concentrations of the PolyP in the extracts were too low to be detected using NMR, samples were concentrated by precipitating the extracts with 2 volumes of isopropanol. NMR analysis of the concentrated samples could, however, still not confirm the presence of PolyP in any of the transformed plant samples (Figure 4B & C). Although some peaks were

observed in the range where PolyP would be expected at 20 (Figure 4B, PolyP$_i$4 and Figure 4C, PolyP$_i$4), 20.2 (Figure 4B, PolyP$_i$2-2) and 20.7 (Figure 4C, PolyP$_i$2-2), these peaks were not close enough to the PolyP control values to be considered belonging to PolyP or were corresponding to the ATP control (20.7). The presence of PolyP could therefore not be conclusively determined with this technique.

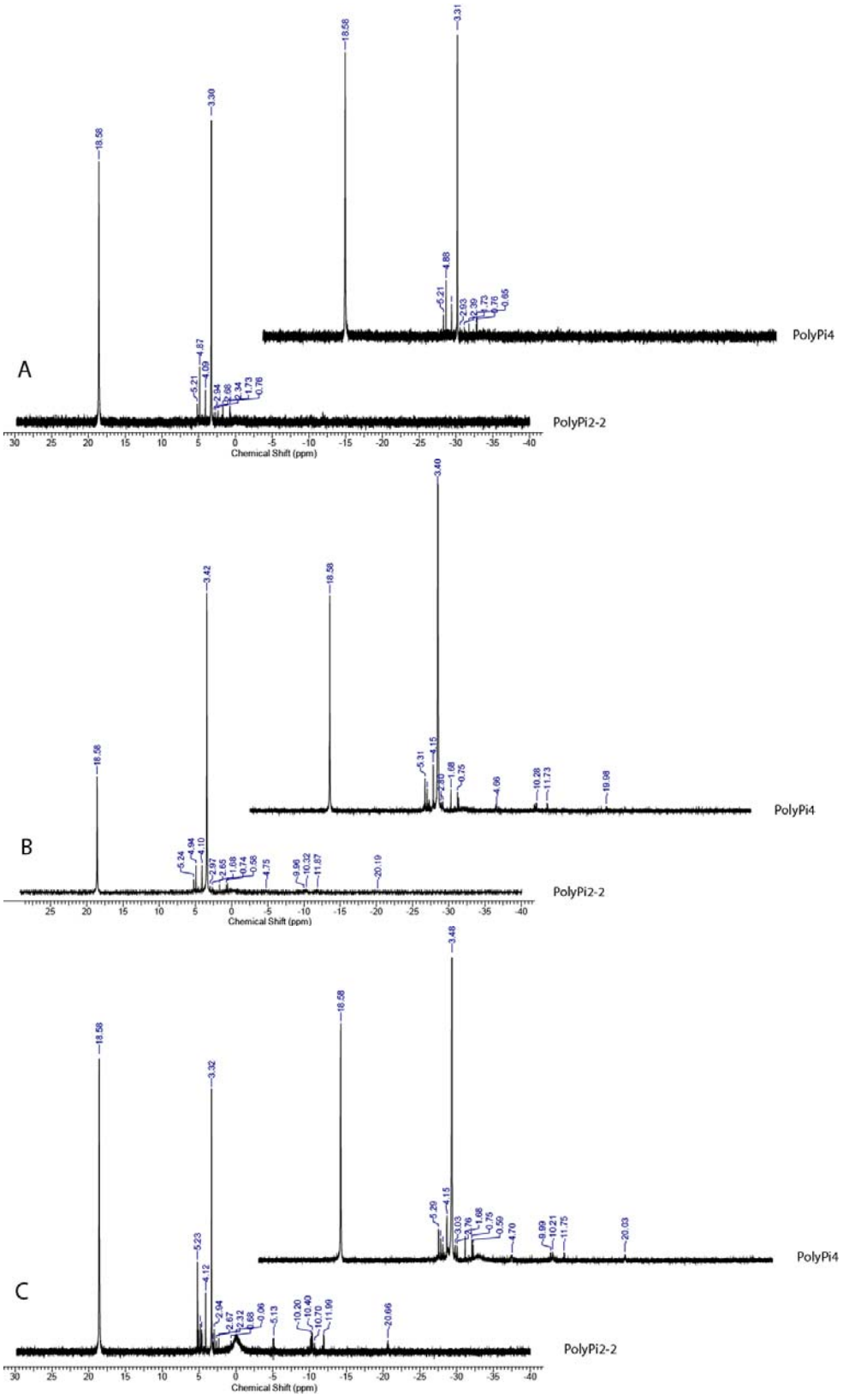


Figure 4. NMR spectra of *Arabidopsis* plant and callus extracts transformed with PHM2, 3 and 4.

All spectra were referenced to MDP (methylene diphosphonate) as external reference in a capillary insert at a chemical shift of 18.58 ppm. **A** was the spectra obtained for unconcentrated callus extracts and **B** and **C** was the spectra obtained for concentrated plant and callus extracts respectively.

Although the concentrations of the novel PolyP product produced by the plant could have been too low for detection using NMR, its presence may still be confirmed by digesting it with specific enzymes and detection of any changes occurring in the substrate. We therefore digested the extract samples with the yeast PPX enzyme that specifically degrades PolyP (34). The digested and undigested samples were then again analysed using PAGE and stained with toluidine blue (Figure 5). The staining of the yeast extracts showed a dark purple colouring with a dark blue undertone that vanished upon treatment with nucleases. This confirms the blue colour of the nucleic acids obtained with this staining method. When the yeast samples were however treated with the PPX enzyme all the stained compounds disappeared from the gel, demonstrating the effect of the PPX enzyme. Although the intensity of the smear detected on the gel for the plant callus extracts were much lower than that detected for the yeast extracts, it was still clearly observed on the gel for the transformed lines, but not for the wild-type callus extracts. Similar to the yeast control treatments the purple smears on the gel for the transgenic lines were unaffected by the nucleases but vanished completely upon treatment with the PPX enzyme. It was, therefore, clear from the result that the polymer formed by the transformed plant lines was indeed PolyP (Figure 5).

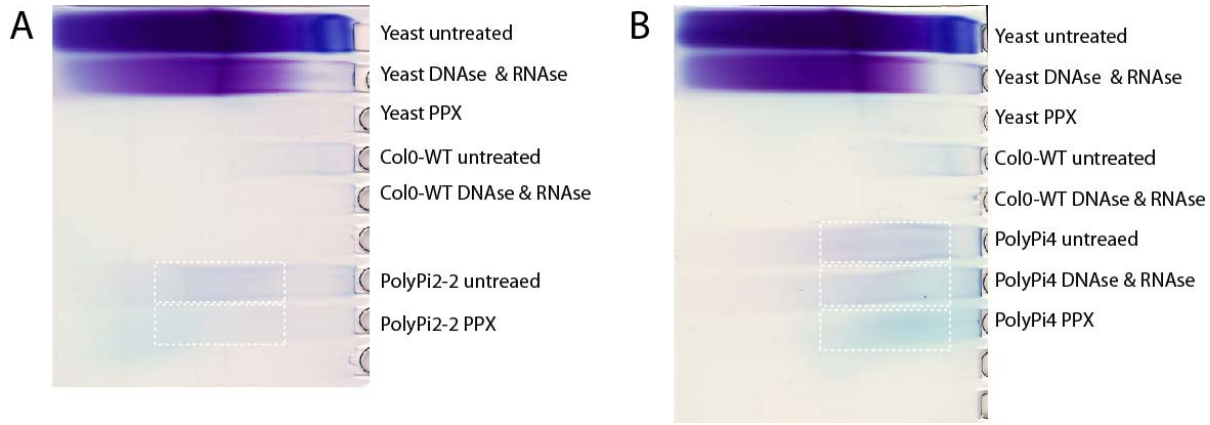


Figure 5. PAGE analysis of plant extracts treated with PPX and stained with Toluidine Blue.

A is the precipitated extracts of the PolyP$_i$2-2 clone callus and **B** of the PolyP$_i$4 clone. Samples were either untreated or treated with only DNAse and RNAse or treated with DNAse, RNAse and PPX. The observed changes are indicated by the white dotted line boxes.

In this study we attempted to functionally express the identified membrane bound enzyme complex from yeast in plants in such a way that it would introduce the production of PolyP in the plant vacuole. The level of PolyP produced by the transgenic plants was extremely low and could not be quantified using NMR. The NMR technique could effectively identify PolyP in yeast cells and could identify added commercial PolyP that was added to the wild-type plant extracts at a concentration of 3 mg.ml^{-1} indicating that the concentration of PolyP in the concentrated extracts were lower than that and probably in the µM or nM range. Considering that the concentrated samples were obtained by precipitating extracts from 10g of plant material that means that the concentration of PolyP in the

plant material was probably in the low nM to pM concentration range. The presence of this polymer was, however, conclusively confirmed after PPX enzyme treatment and loading of the complete extract and enzyme treated samples on PAGE and toluidine blue staining.

The detection of PolyP in the transgenic callus and its absence in the wild-type proves that introduction of the PHM2, 3 and 4 genes was sufficient to produce PolyP in plant cells. The correct targeting to the vacuole, on the other hand, has not been confirmed. Ideally, the sub-cellular detection of PolyP by histochemistry would be the technique of choice to prove that PolyP was indeed targeted to this compartment. Since the levels of PolyP production in the transgenic plants was very low, and would not allow for a perturbation of the sub-cellular P_i compartmentalisation, further investigations with the transgenic plants were not feasible and therefore terminated.

The unusually long discovery cycle observed with the elucidation of the mechanism of this enzyme complex emphasises the difficulty in studying membrane bound enzymes and underlies the importance, as well as our lack of understanding of this critically important field of membrane bound enzymes and membrane trafficking. The compartmentalisation of metabolic processes in eukaryotes is the main feature that sets it apart from prokaryotes and therefore emphasises the importance of the membrane and associated proteins in eukaryotic cells.

The understanding of the targeting and correct processing of membrane bound enzymes is an important first step in our elucidation of this very fundamental part of cell biology. The targeting of membrane bound proteins between species is then also one way of trying to understand the differences between the species in question. The finding that the PHM complex can indeed be functionally expressed in plants in its native form (without removing targeting) therefore brings us a small step closer to understanding the interspecies targeting process. The complex was however only producing PolyP at very low concentrations, but still showed enough activity for the product to be detected. Although we failed to accomplish our main goal of perturbing P_i concentrations for the purpose of investigating P_i homeostasis, this is certainly still an important discovery and one step closer to the understanding of the very complex differences between unicellular and multicellular eukaryotes.

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-Chapter 5-

FRET nanosensors towards unravelling the dynamics of P_i homeostasis in multi-cellular plant organs.

Introduction

Phosphorous (P) is an essential macronutrient, which is required by plants for a wide variety of processes and is a constituent of many structural and functional macromolecules. Plants are sensitive to the form of P that it can assimilate and needs P to be in the form of inorganic P or orthophosphate (P_i) for it to be effectively assimilated. P_i is found in plants as part of nucleic acids, phospholipids and ATP, and is also involved in energy transfer, the activation of proteins and the regulation of metabolism through phosphorylation of intermediates (1). ATP is used as the main energy storage metabolite in the cell and acts as an 'energy currency' for most cellular processes. The hydrolysis of ATP to ADP releases energy that is again utilised by other processes in the cell. In fact, ATP is required for the activation of glucose to allow it to enter the glycolytic pathway. It is thus clear that P_i is a critical metabolite of which only subtle changes in concentration in a specific cell type or sub-cellular compartment can result in a severe effect on cell metabolism and growth.

P_i and phosphorylated metabolites are present in all sub-cellular compartments at varying concentrations (2). These concentrations can differ significantly between various cellular types within a specific tissue. Until recently, no techniques existed that permitted quantitative determination of P_i concentration changes in living cells and sub-cellular compartments. Non-aqueous fractionation is not dynamic, has no resolution to the cellular level and is invasive and sensitive to artefacts. ^{31}P NMR and other spectroscopic techniques such as positron emission tomography is dynamic but has poor spatial resolution (3) and ^{31}P NMR is limited to the millimolar concentration range and can only be used where the pH differs between compartments (2).

Recently, however, a highly sensitive technique based on genetically expressed Förster resonance energy transfer (FRET) sensors that allow for the dynamic analysis of P_i in sub-cellular compartments, was developed by Gu *et al.* (4). Several reviews covering this technology and its comparison to other techniques were published and can be consulted for detailed discussions (3, 5-11). The P_i sensor in particular, was tested on Chinese hamster ovary (CHO) and COS-7 cells and it was demonstrated that these sensors are capable of detecting real-time changes in P_i concentrations in living cells. Affinity mutants of these sensors with the ability to detect P_i over a wide concentration range were also developed. One of the appealing aspects of this technology is the fact that it can make a distinction between the individual cells in a particular organ, allowing the dynamic tracking of P_i concentrations

across multiple cells and tissues of particular organs such as roots and shoots. Resolution of this kind was not available before (5).

This technique opens up new possibilities for the analysis of P_i acquisition by plant roots, allowing for the first time the dynamic tracking of P_i with cellular and sub-cellular resolution. This can constitute a first step towards the understanding of the dynamic processes of P_i uptake and homeostasis. Until recently, this technique has, however, only been used on the single cell level and not on organs where multiple cells could be investigated simultaneously. A recent study by Chaudhuri *et al.* (2008), where glucose and sucrose concentration changes in the root tips of *Arabidopsis thaliana* were analyzed, have laid some of the ground work for these kinds of studies in plant roots (12). However, concentration gradients across the multiple cells of the root tip were not observed. In another study by Deuschle *et al*. (2006) the same sensors were used to study glucose induced FRET changes in the cytosol of *Arabidopsis* leaf epidermal, guard and root cells. It was found that cytosolic steady state glucose levels are dependent on external supply in both tissues and in non-photosynthetic conditions the glucose levels can drop to lower than 90 nM concentrations in root cells (13). P_i is transported by the plant roots to the rest of the plant by various transporters and specialised cell types such as the vascular bundles, and it would be interesting to observe the dynamic flux of P_i across the plant root during phosphate uptake and redistribution.

In this study the newly developed P_i FRET sensors were utilized to address the challenging question of how the P_i concentration changes across the multiple cells and tissue layers of the maturing plant root during phosphate uptake. It is demonstrated that the cytosolic P_i concentrations of P_i starved *Arabidopsis* roots are largely resistant to outside changes in P_i concentrations in the concentrations ranges investigated. None of the sensors employed could effectively measure any definite changes over the time periods investigated. Small fluctuations could however be detected in the low µM to nM range and here a large P_i concentration gradient exists across the plant root. It seems that a change in concentration of P_i in one part of the root influences the P_i concentration in other parts of the root. More sensitive affinity mutants than the ones used in the present study would however be needed to make quantifiable measurements. This observation confirms recent findings in literature that P_i concentrations in the plant cytoplasm is in fact far lower than previously expected and drops to below 15 µM when the plants are under P_i limitation (14). It also emphasises the impossibility to average concentration readings across the whole root and demonstrates clearly the importance of improved resolution to truly understand the dynamics of P_i homeostasis.

Materials and Methods

Plasmid construction and bacterial transformation: Three affinity mutants of the FLIPPi nanosensors (4) cloned into the pRSET-B bacterial expression vector (Invitrogen, USA) FlIPPi-30m, FlIPPi-200µ and FLIPPi-4 µ were received from the Department of Plant Biology, Carnegie Institution for Science, Stanford USA. DH5α cells were transformed with the

constructs and amplified. To allow expression in plants, the sensors were subcloned into pCambia 1300 (http://www.cambia.org). The modified CAMBIA vector was constructed as follows. The expression cassette of pBinAR-kan (15) was cloned into the *Eco*RI / *Hin*dIII restriction sites of pCAMBIA 1300, replacing the multiple cloning site. To insert the nanosensor into the resulting plasmid (pCAMBIA 1300 - Bin) it was first digested with *Bam*HI and *Hind*III for subcloning into pBluescript, after which the inserts were removed by restriction digestion with *Bam*HI and *Sal*I and ligated into pCAMBIA1300-Bin digested with the same enzymes (Figure 1). Inserts were sequenced to verify correct insertion. Electroporation was used to transform the GV3101 *Agrobacterium tumefaciens* strain using the Gene Pulser Xcell Electroporation System (Bio-Rad), following the methods described in the manufacturer's instruction manual.

General plant growth and transformation: Homozygous *Arabidopsis rdr6* lines (16) (donated by Scott Poethig, University of Pennsylvania), were used throughout this study for all transformations and experimental procedures. For transformation, plants were grown from seeds in soil under a 16 hour light, 8 hour dark cycle at 200 µmol $m^{-2}sec^{-1}$ until they started to flower. At this time the flower bolts were removed and allowed to resprout to allow for the formation of more flowers. Plants were then grown under the same conditions until flower bolts reached a height of approximately 200 mm. *Agrobacterium* transformed with the various sensor constructs were used to genetically transform the plants using the method described by Clough and Bent (17). After transformation, plants were grown under the same conditions until seeds started to dry, at which time watering was stopped and the plants left to dry in the growth room. After drying, the seeds were harvested and transformants were selected on solidified (0.8% (w/v) agar) MS media (18) pH 5.7 containing 50 $\mu g.ml^{-1}$ Hygromycin and 3% (w/v) sucrose. Positive seedlings were then hardened off and screened for YFP fluorescence using a Leica MZ10 F epifluorescence stereo microscope. Plants with the highest level of fluorescence were selected and their seeds were collected. These seeds were in turn germinated on soil and screened for YFP fluorescence. Plants with high levels of fluorescence were once again selected for seed collection. These seeds (T_3 generation) were pooled for each sensor line and used in further experiments.

Plant growth and selection for sensor experiments: For imaging experiments, plants were grown on ½ strength MS media (18) with 3% (w/v) sucrose, 20mM MES pH 5.7, solidified with 0.8% (w/v) agar, pH adjusted with KOH. P_i concentrations in the media were adjusted by adding specific molar concentrations of KH_2PO_4. Plates were incubated in a growth chamber with 16 h light at 200 µmol $m^{-2}sec^{-1}$, at 23˚C for 7 - 10 days. The sensors were previously characterised *in vitro* and in CHO and COS-7 cells, but have not been applied to plants to investigate *in vivo* P_i concentrations. In order to examine the cytosolic P_i levels, seedlings were screened for fluorescence and the plants with the highest levels of fluorescence were selected for the experiment a day before the perfusion experiments. These plants were placed in the same media but without the addition of sucrose and solidifying agent to allow plants to acclimatise for perfusion experiments. Seedlings were incubated under the same conditions for 12 h until the onset of perfusion experiments.

Perfusion experiments: Selected seedlings were immobilised on 30mm CellStar tissue culture dishes (Greiner Bio-One catalogue no. 627160) using medical adhesive as described in (12). For initial experiments to determine sensor response, seedlings were perfused with ½ strength MS with 20mM MES pH 5.7 without P_i for 5 min at 3 ml min^{-1} by making use of a Gilson peristaltic pump. After 5 min, buffer supply was changed to ½ strength MS with 20mM MES pH 5.7, including a near-saturating concentration (depending on the specific affinity mutant used) of KH_2PO_4 and perfused for a further 15 min at 3ml min^{-1}. Plants were imaged for the full duration of the perfusion experiment. FRET was determined using an IX-81 Olympus microscope, equipped with a F-view-II cooled CCD camera (Soft Imaging Systems) coupled to an MT-20 (Soft Imaging Systems) xenon arc lamp, by excitation using FRET excitation filters (S430/25x and S500/200, Chroma). Fluorescence emission was monitored at one frame per 10 seconds for 20 min experiments and one frame per 7.5 minutes for overnight experiments, by collection windows for CFP emission and YFP emission (S470/30m

and S535/30m, Chroma respectively). CFP/YFP ratios were calculated using the Olympus Cell^R package. Through setting up a defined experiment in the Experiment Manager feature of the Cell^R software, image acquisition parameters such as exposure time, illumination settings and emission filter cube selection were kept constant for all groups and ensured appropriate selection of parameters. Accumulation rates for P_i was determined by calculating changes in intensity ratio (535/470 nm) over time for various external P_i concentrations. Regions outside the root area were used for background subtraction.

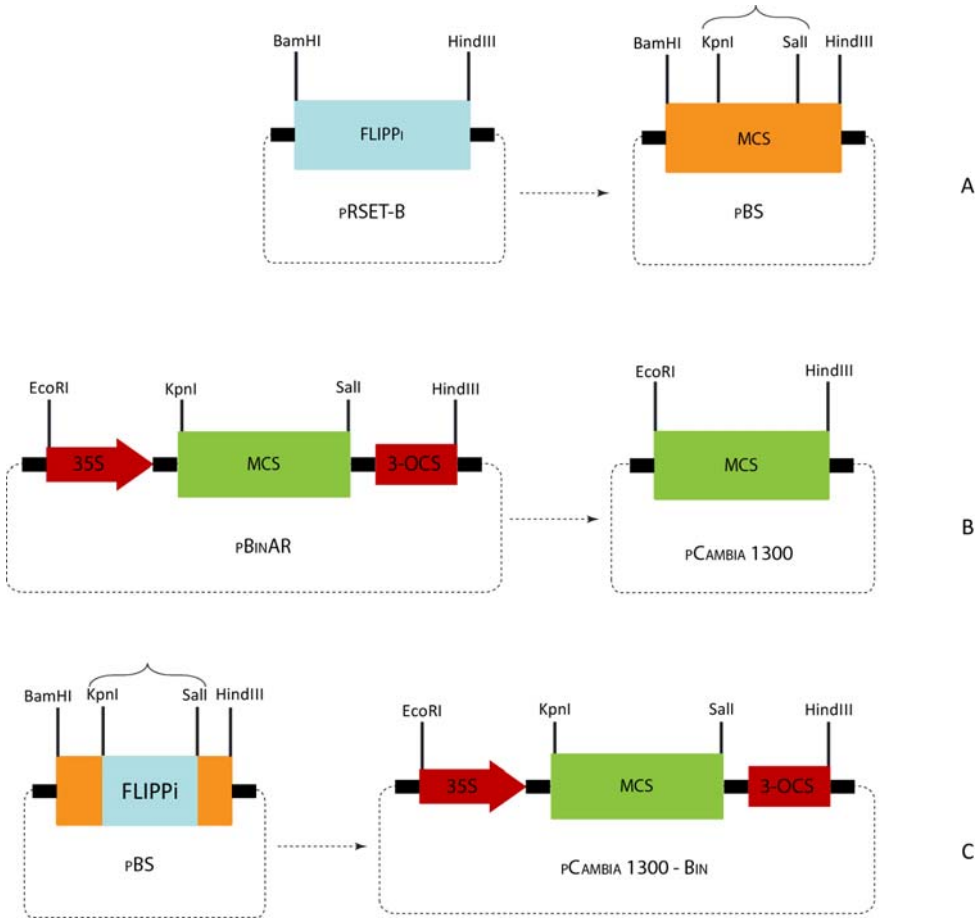


Figure 1. Vector construction and subcloning of FLIPPi into pCAMBIA 1300 – Bin

(A) FLIPPi nanosensors cloned in pRSET-B vector were cut with *Bam*HI and *Hind*III restriction enzymes and cloned into the corresponding sites of pBluescript for subsequent digestions.

(B) pCAMBIA 1300-Bin was constructed by cloning the expression cassette of pBINAR into the *Eco*RI / *Hind*III positions of the multiple cloning site of pCAMBIA 1300, replacing it completely.

(C) FLIPPi constructs were then subcloned from pBluescript into the new pCAMBIA 1300 – BIN plasmid using the *Kpn*I and *Sal*I restriction sites.

Results and discussion

To critically evaluate our knowledge about the processes involved in phosphate homeostasis we need to be able to measure P_i concentrations in a wide range of cellular types and sub-cellular compartments. To experimentally determine flux we also need to be able to follow the movement of P_i (changes in concentration) across cell types and compartments. Current quantification techniques have limited capabilities in this regard and new, less invasive approaches with greater resolution are

required. Therefore, in the current study we set out to evaluate one of the most promising new techniques that was recently developed (4) for its capabilities of fulfilling these needs. The FRET nanosensors that we tested had already proven to be extremely powerful when used for this purpose on a wide range of other metabolites and in other systems. However, these nanosensors have not been used to investigate metabolite flux in and between plant cells in larger organs with various cell types of different function. Although Deuschle *et al.* determined the glucose concentrations in pavement and guard cells of *Arabidopsis* leaves (13), the movement of metabolites between these cells were not discussed. The P_i specific nanosensors have also not yet been applied to plant cells. In order to study the P_i concentration in the cytosol of living *Arabidopsis* roots we transformed these plants with the various affinity mutants of the P_i sensor, expected to match the anticipated P_i concentrations in the plant cytosol.

Expression of P_i affinity mutant FLIPPis in *Arabidopsis* rdr6 mutants.

Due to the previously observed post-translational silencing of similarly constructed nanosensors when it was tried to express them in plants (13), transformations were done in *rdr6* mutants defective in the RNA silencing pathway. After transformation and kanamycin selection, seedlings were screened for eYFP fluorescence and only those showing the highest level of fluorescence were allowed to mature for seed collection. Wild-type plants showed no fluorescence, while the transformed plants had a large variation in the level of fluorescence. While leaves showed some fluorescent activity, roots in general had higher levels. Fluorescence levels were not determined but only visually selected. The subsequent generation of plants was again screened for maximal fluorescence and the T_3 generation was used for all subsequent experiments.

In order to allow the plant to upregulate its P_i transporting machinery, the transformants were grown under conditions of P_i depletion. The plants were then imaged while P_i was supplied and the P_i concentration dependant FRET changes recorded.

The recording of fluorescence data from growing plant roots over a number of hours presented us with several challenges, especially with regard to the movement of cells during the recording phase, rendering the recorded data not interpretable. Plants were actively growing during the experiments and the expanding and dividing root cells caused the area under observation to change, with the result that the various time points recorded could not be compared. The experimental setup used was similar to that described by Chaudhuri *et al*. (12). This setup proved to be remarkable at holding the roots in place and worked very well for the short time lapse experiments (up to 30 min), but when the time lapse experiments were extended to several hours, the growth of the root cells started to interfere with the measurements. In order to overcome this problem, we repeated measurements with seedlings of various ages and growth stages. At first, 3 - 5 day old seedlings were used, but during this stage the

root growth rate was extremely high and the data could not be used. When slightly older plants were chosen, (7 – 10 days after germination), movement was considerably less and allowed to complete enough experiments to allow for enough data to be collected. At 10 days after germination the root growth had slowed down to allow uninterrupted measurements to be done over 12 – 15 hours.

Cytosolic P_i concentrations appear to be resistant to change, with only small ratio changes observed.

Root tips of *Arabidopsis* plants have previously been used in experiments to study glucose and sucrose concentration changes by making use of an affinity series of glucose and sucrose sensors (12, 13). Concentration dependant ratio changes in the cytosol of individual leaf epidermal cells were also measured in an independent study by the same group (13). These principles and methodology were used in the current study and it laid the groundwork for the current investigation of P_i concentration dependent changes the ratio of fluorescence between donor and acceptor fluorophores in *Arabidopsis* roots. *Arabidopsis* plants stably transformed with three affinity mutants of the P_i sensor developed by Gu *et al.* (4) were used and the details of these sensors are summarised in Table 1.

Table 1 affinity mutants of the FLIPPi nanosensor

FLIPPi affinity mutants			
Sensor	**K_d (M)**	**ΔR_{max}**	**Dynamic range**
FLIPPi-4µ	3.9×10^{-6}	-1.34	0.4–25 µM
FLIPPi-200µ	2.1×10^{-4}	-1.13	25–1600 µM
FLIPPi-30m	3.3×10^{-2}	-1.03	3–170 mM

See (4) for details

Plants transformed with the various affinity mutants were grown on solid half strength MS media without the addition of P_i and perfused with near saturating concentrations of P_i, but none of the transformants investigated showed any significant ratio changes over the 20 min time span of the experiment (Figure 2).

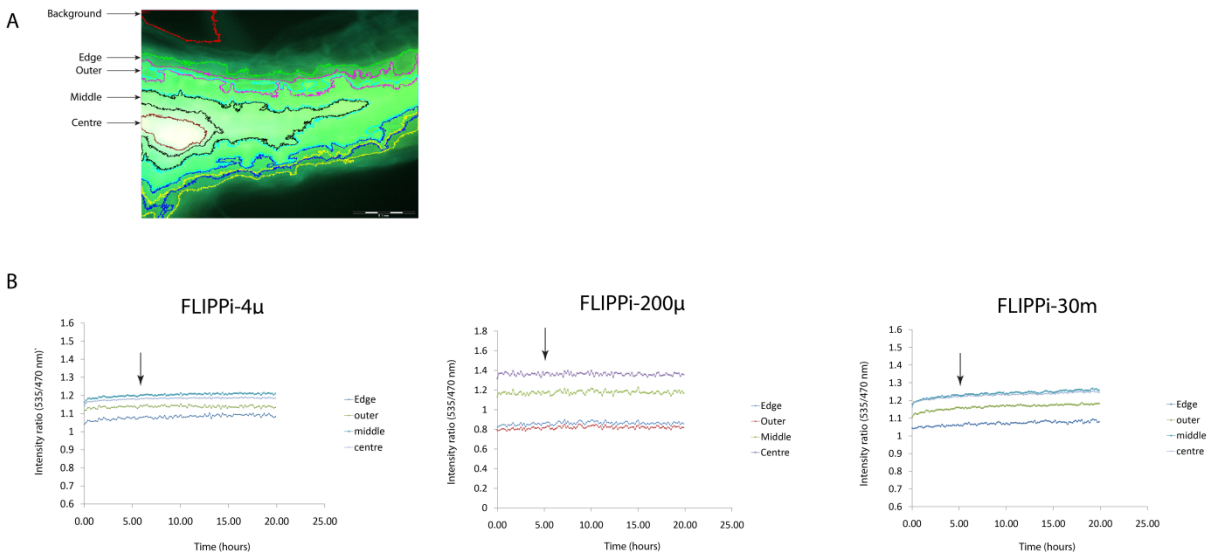
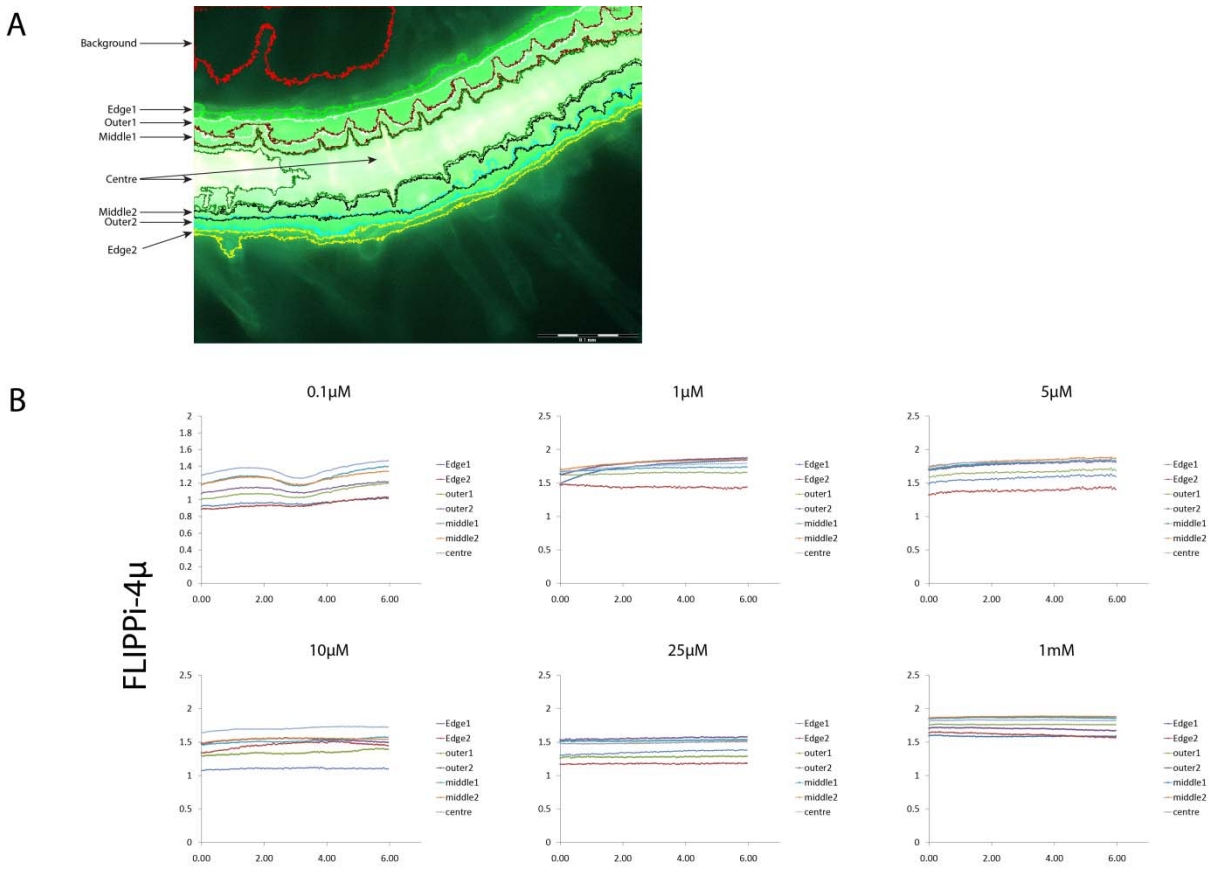


Figure 2. Ratiometric FRET changes of *Arabidopsis* roots perfused with saturating concentrations of P_i over a 20 min time span. **(A)** Flourescence image of an *Arabidopsis* root, showing selected regions of interest (ROIs). **(B)** Measured intensity ratios for all ROIs for the individual affinity mutants over 20 min. Roots were perfused with half strength MS, without the addition of P_i for the first 5 minutes, whereafter the perfusion media was changed to half strength MS containing 25µM P_i for FLIPPi-4µ, 6mM P_i for FLIPPi-200µ and 170mM P_i for FLIPPi-30m.

At first, the absence of any observed ratio changes for all the sensors over a 20 min time period was interpreted to denote that the sensors were, for some reason, inactive in the plants. However, when the experimental time was extended to several hours, ratio changes started to emerge for the high affinity sensors (Figure 3). When *Arabidopsis* roots transformed with the FLIPPi-4µ construct and grown under P_i depleted conditions were perfused with micromolar concentrations of P_i (see figure 3 B, C, and D for the specific concentrations used), a marked decrease in intensity ratio (indicating an increase in P_i concentrations) was observed, starting at around two hours after the addition of P_i. The ratio decreased over time for about 90 min and then switched back and started to increase again over time. However, when roots were perfused with higher concentrations of P_i, the pattern of the observed ratio changes was altered so that some regions of interest (ROIs) showed considerably larger changes than others. While some ROIs demonstrated an increase in ratio, others showed a decrease (Figure 3). When exposed to higher P_i concentrations however most ROIs remained constant and far less changes could be observed.

The same result was observed when roots were perfused with the new P_i concentrations than when the media was simply changed by hand to the new concentration and recorded over time. Because of the extended time span of the experiments and the large amount of media subsequently required, the long time lapse experiments were therefore carried out by simply replacing the low P_i media with media containing the new P_i concentrations. Ratio changes stabilised after five hours in most cases and

experiments were therefore carried out over 6 hours with a range of P_i concentrations, starting below the detection range of FLIPPi-4μ and ending above the detection range of FLIPPi-30m (Figure 3).



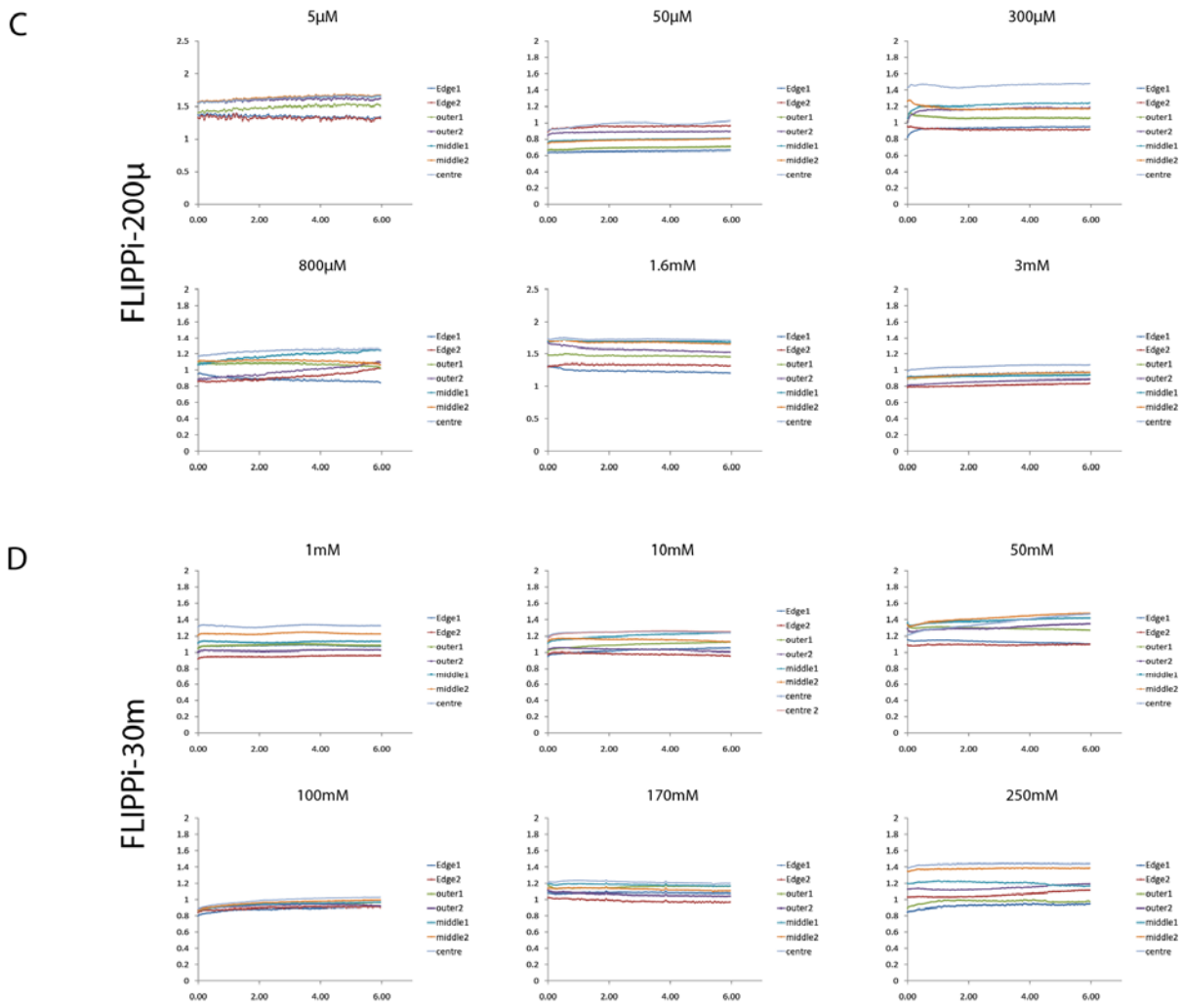


Figure 3. Observed FRET ratio changes in *Arabidopsis* roots transformed with FLIPPi nanosensors.

In all instances the y-axis represents the measured intensity ratio changes (535/470 nm) and the x-axis represents time in hours. (**A**) Fluorescent image of *Arabidopsis* root expressing FLIPPi nanosensor and indicating the various ROIs selected for analysis. Changes in intensity ratios observed for the (**B**) FLIPPi-4µ sensor, (**C**) FLIPPi-200µ sensor and (**D**) Graph FLIPPi-30m sensor. Specific concentrations used in the perfusions are indicated above each graph in **B**, **C** and **D**.

The absence of ratio changes could have several explanations and can be interpreted in various ways. The first possible interpretation is that, although they showed strong fluorescence, these sensors were inactive in the plants and did for some reason not react to the changing P_i levels in the root cell cytosol. On the other hand, the plant could have been extremely effective in buffering the changes in P_i concentration that was occurring in the cytosol, to such an extent that it was preventing it, which resulted in zero absolute change. Plants have several mechanisms of buffering P_i concentrations, of which the transport to other compartments is probably the most prominent. This process would probably take place at a similar rate than the import of P_i into the cytosol and this would therefore still be detected by the sensor. Other mechanisms like the strong binding of P_i to P_i scavenging compounds or proteins could, however, account for the apparent absence of P_i concentration changes, especially if this happened during import of P_i into the cell. The fact that the plants were subjected to P_i

stress conditions prior to the perfusion experiments would suggest that very low P_i concentrations were present in the cytosol at the onset of the tests. This could then in theory have resulted in the over-expression of P_i scavenging compounds that could easily buffer the P_i concentrations in the P_i depleted cells during uptake and the plant would keep the entire available P_i in a bound form, inaccessible to the sensors and thus not detected by them.

Another explanation for the observed absence in P_i ratio changes would be that there just were no changes occurring, and that P_i import into P_i starved *Arabidopsis* root cells is very slow. This explanation could be true to a certain extent because it is known that high affinity P_i transporters that are expressed during P_i stress conditions utilised in the experiments are also slow transporters due to the high affinity of the transporters for P_i. The time frame is however, quite long and this would be unlikely but still a possibility. The fact that later experiments (Figures 3 and 4) then also indicated a change after several hours, also in part supports this notion.

Another possible explanation that no P_i ratio changes were observed is that P_i transport in plant roots under these P_i limiting conditions may be happening in such a way that it never passes through the cytosol of the cells. P_i may thus be directly transported to the vascular bundles or vacuoles through micropores and the apoplastic space after it has been imported by the transporters. The nanosensors may therefore not have been expressed in the compartment where the P_i accumulation was taking place.

One other interesting observation was the results obtained for FLIPPi-30m. In this instance, the measured ratio increased slightly over the time period, signifying a decrease in P_i concentrations (Figure 4 (III) A). This observation is difficult to fathom and it was seen as experimental error, but the experiment was repeated several times with different plants and the same results were obtained for all of these. The only explanation for this observation is that either the introduction of P_i into the P_i starved system was sensed by the plant and in response some P_i storage mechanisms were activated, reducing the available P_i in the cytoplasm or, more likely, that the introduction of P_i caused the intracellular environment to change to such an extent that it changed the conformation of the sensors resulting in a non-specific ratio change.

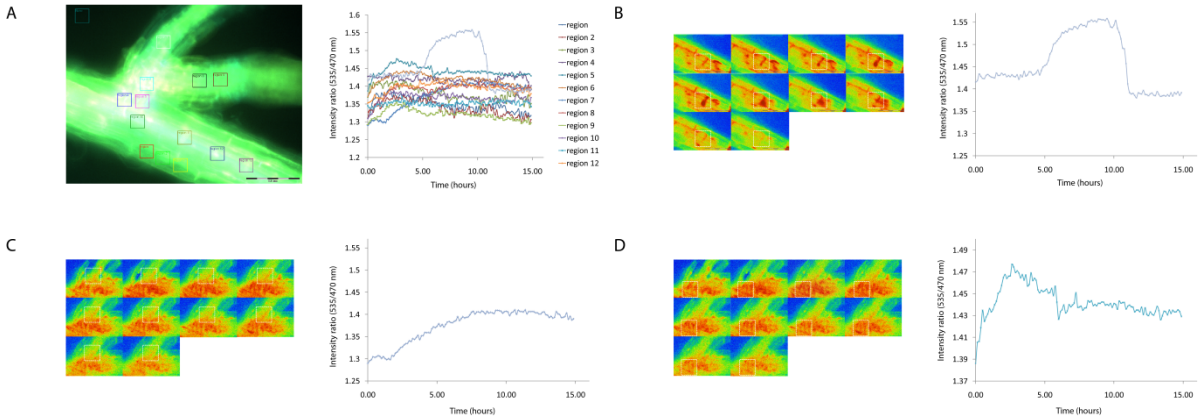
In subsequent experiments, however, the experimental time was extended to several hours and then it was possible to observe definite changes in measured FRET ratios. This, on first glance, supports the hypothesis that P_i transport into the cytoplasm is extremely slow and takes several hours to increase P_i concentrations to the point where it can be detected by the sensors. One other trend that started to emerge from the data collected from the longer time lapse experiments, were that discrepancies were observed between the observed ratio changes for different areas of the root. When looking at Figure 3 B, this observation is evident from the 0.1µM and the 1µM concentration measurements and also to a certain extent in the other measurements. No real pattern, however, could be determined from these

observations, except maybe that the ratio changes were more pronounced and uniform at the lower concentrations. With the 0.1µM concentration, it is also interesting to note that the apparent increase in concentration was more pronounced at the centre of the root and the vascular bundles and was less evident the farther the measurements was taken from the centre. This would be expected if the plant concentrated P_i in the vascular bundles in order to transport it to the rest of the plant. What was also interesting to note was that the initial increase in concentration observed in the first part of the experiment was reversed in the second part and the concentrations returned to their initial values. This can easily be explained in that the low P_i concentrations supplied was exhausted by the plant and therefore the internal concentrations returned to their initial values. What is however unclear is why the initial observed increase in P_i concentrations is not repeated when the supplied P_i concentrations are raised. One possible explanation for this could be that P_i starved plants can sense P_i concentrations on the outside of the root and as soon as these concentrations reach a certain level the plant switches to a different transport mechanism that is not detectable by cytosolic P_i sensors. In his recent review, Doerner (2008) also discussed the existence of P_i sensing capabilities of the plant involved in the upstream activation of miR399 and IPS/AT4 signalling in relation to P_i uptake (19). The sensing of P_i is needed for these signalling mechanisms to work, and perhaps this signalling module is also linked to a different mode of P_i uptake under low and high P_i concentrations, other than the mere expression of high affinity P_i transporters.

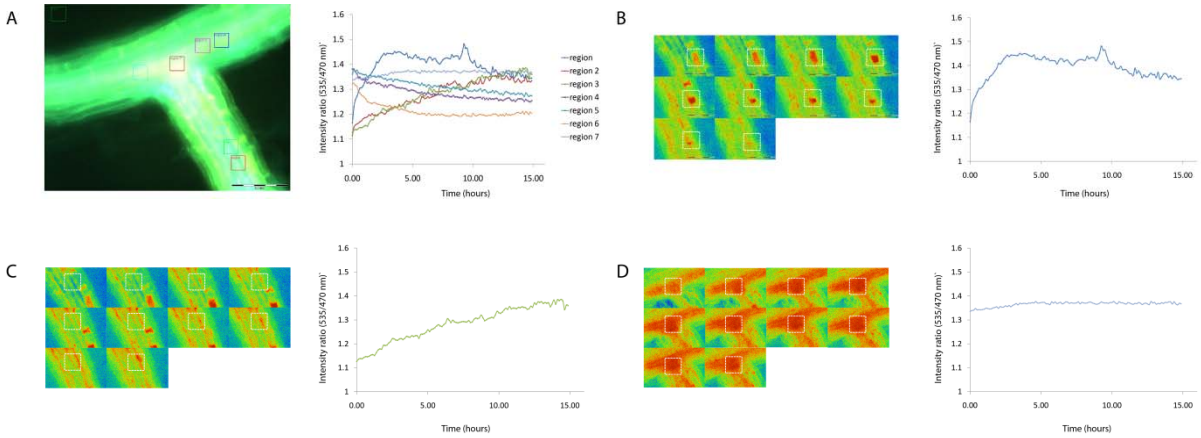
Large variations in P_i concentration observed across the root with the choice of smaller regions of interest.

During the analysis of the time lapse experiments the regions indicated in Figure 3 were chosen in order to try and group similar cells together on the basis of their distance from the edge of the root and from the vascular bundles in the centre. Using this approach, only small changes in the ratios over these areas were observed. However, when ratiometric timeframe videos of these experiments were subsequently examined (Figure 4), it was observed that some ratios were indeed changing, but that these changes happened in very specific and localised areas of the root. New time lapse experiments that stretched over even longer periods and focussed on much smaller, more defined ROIs were therefore initiated. The results of these experiments were quite different from the results observed with the larger regions and showed great variation in the P_i concentrations in various parts of the roots expressing the FLIPPi-4µ and FLIPPi-200µ sensors (Figure 4). The most prominent ratiometric changes in the localised areas are shown in the screen captures of the selected ROIs (Figure 4 (I) B – D and (II) B – D). No differences could, however, be observed between the selected regions of roots expressing FLIPPi-30m, showing a decrease in P_i concentrations for all selected regions. Screen captures were therefore not included for this sensor (Figure 4 (III). During the analysis of FRET ratio changes in *Arabidopsis* roots transformed with the various affinity mutants of the FLIPPi nanosensor, ratio changes detected for specific ROIs showed considerably different values and trends for the low affinity sensors.

(I) FLIPPi-4µ



(II) FLIPPi-200µ



(III) FLIPPi-30m

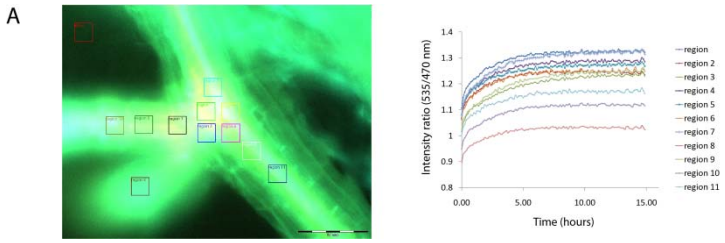


Figure 4. Changes in FRET intensity ratios observed in localised regions of the *Arabidopsis* root.

(**I**) – (**III**) Ratio changes detected for the FLIPPi-4µ, FLIPPi-200µ and FLIPPi-30m sensors respectively, with **A** in all cases showing the selected ROIs for the individual experiments (full resolution images and videos can be obtained from the online supplementary material) together with a graph of all the ROIs selected for that experiment. **B** – **D** is in turn showing the various ratio changes for three selected ROIs from each, together with screen captures of the ratiometric images taken from selected time points during the time lapse experiments.

These findings confirm the importance of cellular resolution when metabolite concentrations and flux are measured. What is quite surprising, is that the sharp peaks observed in many of the experiments

signifies a decrease in P_i concentrations in these areas, while the roots were actually supplied with an increased P_i concentration on the outside (Figure 4 (I) B and D and (II) B). For some reason P_i concentrations decreased sharply in these areas or the sensors were for some reason adversely affected in those areas, causing non-specific ratio changes. It is suggested, that these areas are evidence of some P_i sensing and signalling mechanism by the plant. The plant senses an increase in P_i concentration on the outside and then upregulates its transport mechanisms to the shoots, leading to a decrease in P_i concentrations in some of the root cells. This concentration gradient is then again quickly abolished when it reaches a certain level (This result can be observed in the screen captures provided in Figure 4 (I) B – D and (II) B – D).

The results obtained here support recent findings by Pratt *et al*. (2009) who employed an improved NMR technique to demonstrate far lower P_i concentrations in the cytoplasm (60 - 80 µM) than what was previously shown. They also showed that the P_i concentrations dropped quickly to lower than 15 µM soon after P_i supply was removed. Considering that experiments in the current study were based on plants grown for more than 7 days without P_i supply, it would not be surprising if P_i concentrations in the cytosol was in the nM range and therefore undetectable by the sensors employed. We therefore suggest further investigations with higher affinity sensors.

The findings of this study are opening up many questions regarding the mechanisms involved in P_i uptake and transport to the shoots. The initial suggestion that current techniques lack the required resolution to study P_i transport and flux proved to be correct. Just by applying these sensors in a very broad manner as we did, some new findings were already made. Why do we observe no P_i induced ratio changes over short time lapse experiments? Why do P_i concentrations seem to decrease when high P_i concentrations are applied to P_i stressed plants transformed with the low affinity P_i sensor, and what is the significance of the sudden large drop in concentration in some of the root cells? Answers to these questions can only be obtained through further investigation of the P_i uptake mechanisms and currently only with the use of FRET nanosensor technology. We can conclude that the use of FRET nanosensor technology can certainly enhance our understanding of plant P_i transport, and especially in multi-cellular organs like *Arabidopsis* roots. Current findings however did not conclusively show the actual P_i concentrations in these root cells and our specific experimental setup cannot be ruled out as a possible cause. We therefore suggest repeating these experiments with a more similar setup to the published protocol (4) and including plants over-expressing an additional Pi transporter to artificially increase the Pi concentrations in the cytoplasm, making it more detectable.

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-Chapter 6-

Concluding remarks

This work was initiated with several key questions relating to P_i homeostasis in plants and in particular how this process is controlled in the various sub-cellular compartments that makes up a cell. The immense complexity of the reaction networks that are involved in maintaining the P_i balance throughout the plant is evident from the large number of publications that are available in this field. Despite the great scientific interest, it is also evident that several key reactions and proteins responsible for them are still a mystery to scientific investigators. It is for instance still unknown how P_i concentrations are sensed by the plant and how many reactions are involved in this sensing. Especially important is the mechanism of P_i sensing involved in the activation of miR399 and IPS/AT4 gene expression controlling phosphate uptake under P_i limiting conditions (1). Other questions still unanswered relate to the substrates of *PHO2* and how these substrates mediate the over-expression of some of the high affinity P_i transporters (1). We also do not know the mechanism of transport of the miR399 complexes via the phloem. Despite the large number of P_i transport proteins identified to date, evidence exist to support several other transport activities in plants that yet needs to be elucidated. Unexplained activities identified include, export of P_i from amyloplasts not coupled to the transport of phosphorylated organic compounds (2), import of P_i into the vacuole (3) and the export of P_i from the Golgi, where it is generated as a by-product of glycosylation reactions (4). Several discoveries therefore still need to be made before P_i transport and the process of P_i homeostasis can be fully understood.

We decided to focus our efforts on investigating a few key aspects of phosphate homeostasis in plants by specifically looking at 3 ways to investigate sub-cellular compartmentalisation of P_i pools: 1) by elucidating the role of the high affinity P_i transporter PHT1;5 in chloroplasts and photosynthesis under P_i limitation and to thereby perturb subcellular P_i distribution in respective mutants 2) by perturbing the P_i concentration in plant vacuoles by introducing an additional P_i pool in this compartment by expressing the yeast enzyme complex responsible for PolyP production in *Arabidopsis* plants and 3) by evaluating the application of a newly developed group of P_i nanosensors to plant cells to develop tools for the analysis of the mutants and transgenic plants generated, which potentially show changes in sub-cellular P_i distribution.

During characterisation of the role of the PHT1;5 transporter it was found to be localised in plastids and that it significantly contributes to the import of P_i into chloroplasts under P_i limiting conditions. As opposed to other plastidial transporters studied to date, this transporter belongs to the group of high affinity P_i transporters and as such is the first high affinity P_i transporter to be identified in plastids. This

finding is highly significant, especially with regard to photosynthesis under P_i limiting conditions. All other P_i transport in the chloroplasts studied to date was occurring under relatively high concentrations of P_i in the cytoplasm that allowed the low affinity transporters to function. In reality, most plants in their natural habitats needs to grow under P_i limiting conditions and therefore photosynthesis needs to be functioning under these conditions. Our results suggests that plants make use of starch turnover in the light as an alternative mechanism for carbon fixation, keeping P_i levels high enough for ATP synthesis to keep photosynthesis functioning. Under these conditions PHT1;5 is utilised to import P_i into plastids and it is suggested that carbon is exported from the plastid in the form of glucose and maltose, with our results suggesting that maltose is the export product. P_i fertilisation is one of the major sources of agricultural pollution and finding ways to prevent this by improving plant metabolism, helping the plant to be more productive even when supplied with low P_i concentrations is of utmost importance. This finding opens the way for future studies on the improvement of P_i acquisition into chloroplasts, improving photosynthesis under these conditions.

In the next part of the study we successfully expressed 3 genes from yeast in the *Arabidopsis* plant. These genes are naturally targeted to the tonoplast in yeast where it is responsible for the production of PolyP in this organism. PolyP is a polymer of P_i and is the major P_i storage compound in yeast. The complex nature of this enzyme complex and the fact that it is composed of membrane bound proteins, caused that the function of this enzyme was only recently elucidated. In fact we were almost finished with our study when the results detailing the mechanism of this enzyme were first published (5). Previous studies performed on a collection of knockout mutants were used as a basis for our choice of the genes to express in *Arabidopsis* (6). The mechanisms behind sub-cellular targeting and post translational processing of proteins are one of the areas that are still poorly understood in biology, especially the conservation of these mechanisms between species. The finding that 3 genes from yeast can be functionally expressed in plants, suggests that the mechanisms used by plants and yeast have more similarities than what was previously thought. On the other hand we were only successful in detecting the PolyP product in plant callus cells and not in whole plants, pointing towards the breakdown of the product or inactivation of the complex in fully functional plants. This finding is intriguing and we currently do not have an explanation for this observation. The low levels of PolyP produced have then also defeated our efforts to perturb the P_i concentrations in the vacuole, but might point toward some detrimental effect of PolyP on higher plant metabolism. This finding might then also help with future experiments aimed at unravelling the roles and function of PolyP.

During the evaluation of the nanosensor technique we encountered several problems that needed to be addressed in order to allow the successful application of this technique to plants. After these issues were addressed we were able to detect some changes in the P_i concentrations in the plant cytoplasm. The results that we obtained from these studies were, however, confusing and inconclusive and left us with even more unanswered questions. We found that P_i concentrations in the plant cytoplasm were in

essence not changing at all in the short term (20 min) and that the measured concentration changes were only occurring over several hours and at much lower concentrations than was previously reported for this compartment. These results are very confusing and still needs to be clarified by further targeting of the sensor to other compartments, the expression of more sensitive sensors and other perturbations of the P_i concentration in the various compartments. This would allow the evaluation of the unexpected results obtained with this technique and would hopefully clarify these observations, allowing us to fully understand the implications of these findings. In the meantime we tried to explain the results that we obtained by postulating several possible theories for what we think might be responsible for our observations. At least one recent publication (published just after the completion of the current study) suggests that the actual P_i concentration in the plant cytoplasm is far lower than was previously expected (7). This indicates that our findings may indeed be correct and that we at least need to use higher affinity sensors in order to make more meaningful measurements.

For future prospects we suggest utilising the knowledge obtained about activated starch synthesis and degradation pathways to look for clues regarding phosphate homeostasis in plant leaves. The recent elucidation of the 3 component system comprised of PHO2, miR399 and IPS/At4 genes controlling P_i allocation to the shoots might serve as a starting point to search for microRNAs and transcription factors responsible for the regulation of P_i homeostasis in plant leaves. The importance of P_i in plant photosynthesis and the subsequent activation of an alternative set of genes to increase carbon flux through starch in the event of P_i limitation strongly suggest a similar regulatory module present in plant leaves, activating PHT1;5 and the corresponding genes involved in carbon metabolism that we identified in chapter 3. The fact that all the genes of the regulatory module is also expressed in plant leaves would suggest that it is similarly involved in P_i homeostasis in these tissues. Perhaps the answer to this elusive problem lies with the activated genes in carbon metabolism and their regulation.

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