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# A DETAILED STUDY OF IMPORTANCE OF MOLECULAR MECHANISMS IN THE HOMEOSTASIS OF PHOSPHATE

**Physiology**

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## ABSTRACT

Phosphorous in the form of orthophosphate (Pi) 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 Pi in soil is extremely low, therefore all plants in their natural environment are always under some degree of Pi deprivation[4,5.] In periods of Pi limitation the Pi concentration in the cytoplasm drops into the low µM range[6,7]. The low affinity of these transporters for Pi would, however, not allow them to function in soil grown conditions where phosphate is limiting, which is mostly the case in natural ecosystems. This work was initiated with several key questions relating to Pi 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 Pi 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 Pi concentrations are sensed by the plant and how many reactions are involved in this sensing. Especially important is the mechanism of Pi sensing involved in the activation of miR399 and IPS/AT4 gene expression controlling phosphate uptake under Pi 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 Pi transporters (1). We also do not know the mechanism of transport of the miR399 complexes via the phloem. Despite the large number of Pi 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 Pi from amyloplasts not coupled to the transport of phosphorylated organic compounds (2), import of Pi into the vacuole (3) and the export of Pi from the Golgi, where it is generated as a by-product of glycosylation reactions (4). Several discoveries therefore still need to be made before Pi transport and the process of Pi homeostasis can be fully understood.

## KEYWORDS

Phosphate Homeostasis ,Orthophosphate (Pi), Pi Transporters, Polyphosphate

### 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 (Pi) homeostasis (3). The importance of Pi 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 Pi acquisition, endogenous Pi pool sizes and Pi exchange between compartments have been the focus of many studies (4, 1, 5-8). The present study focused on a few key questions surrounding Pi homeostasis in plants.

The process of maintaining Pi 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 Pi homeostasis and phosphate transport is discussed in Chapter 2. The sheer complexity and amount of factors involved in the balance of Pi supply and demand makes it difficult to study the entire process. We therefore focussed our investigation on specifically selected aspects surrounding Pi homeostasis in order to shed some light on the overall picture.

One important aspect adding to the complexity of Pi homeostasis is that Pi is important across all sub-cellular compartments and that the role and concentration of Pi in each of these compartments varies depending on the compartment in question (9). This makes its study extremely difficult. Pi transporters are the proteins largely responsible for maintaining the sub-cellular balance of this anion. To date, five families of Pi 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 31P 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

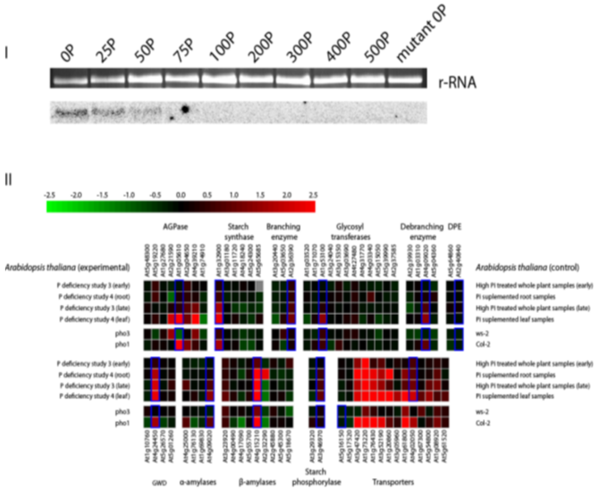

**Figure 1. Expression analysis under Pi limiting conditions.**

**(I)** Northern Blot showing the expression of PHT1;5 when plants are grown with varying concentrations of Pi units are in mg Pi.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 Pi 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 Pi depletion PSII efficiency (ΦPSII), Fv/Fm, 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 Pi supply, no significant differences could be detected between wild-type, mutant and antisense plants (Figure 3 (III)). However, ΦPSII and Fv/Fm were both significantly reduced in the Pi limited, mutant and antisense plants compared to Pi-limited wild-type plants. The decrease in the photosynthetic efficiency indicates a change in the pH gradient across the thylakoid membrane, inhibiting photosynthesis15. This change would most likely be the result of the reduced Pi 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 Pi-limitation, whereas unchanged ATP levels where found in all the different lines growing under sufficient Pi supply or the control lines under Pi-limitation (Figure 3 (IV). This finding supports our hypothesis that PHT1;5 is needed for the import of Pi into chloroplasts under Pi limitation to allow for photosynthetic ATP production. Plants lacking the protein will arrest ATP synthesis, and ultimately photosynthesis, consequently leading to death.

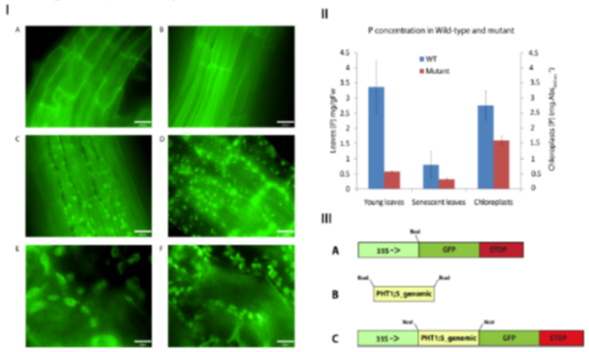

**Figure 2. Sub-cellular localisation of PHT1;5 and effect on Pi distribution under Pi limitation.**

**(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 Pi 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 Pi depletion to allow for the import of Pi 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 Pi –limiting conditions, we made use of the *in silico* gene expression analysis tool Genevestigator16 (https://www.genevestigator.com/gv/index.jsp) to screen for the effect of Pi limitation on the expression of genes involved in carbohydrate metabolism (Figure 1 (II)). 4 Pi limitation experiments were included together with the pho1 and pho3 mutants with lowered leaf Pi 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-workers17 (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 Pi 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 Pi from photosynthetic intermediates when Pi 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 conditions18, which could mean that their expression levels under normal conditions are sufficient to account for all the required export rates.

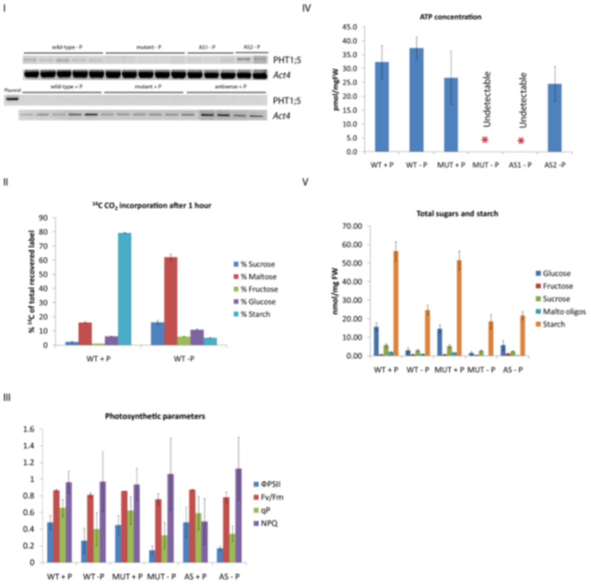

**Figure 3. Metabolic and photosynthetic measurements.**

**(I)** Verification of the expression of PHT1;5 with RT-PCR in plants used for metabolic analysis. **(II)** are 14C CO2 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 Pi-limitation leads to increases in starch turnover, we fed Pi limited wild-type plants with 14C labelled CO2 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 Pi limitation. These results support our hypothesis that Pi 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 Pi depletion, but lower in the mutant plants under Pi limitation than the wild-type.

Some important aspects that would need urgent attention in future experiments would be to experimentally verify the Km values for PHT1;5 for Pi 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 14C 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 Pi 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 0C 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-Kan21 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 method22. 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-2sec-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-2sec-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.

### Pi induction experiments

For selecting the appropriate Pi concentrations that would induce the expression of PHT1;5, plants were grown on vermiculite or palm peat and watered with ½ strength MS salts23 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 KH2PO4, 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 -Pi and +Pi 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 32P 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'-GCTCATGGTACTATGTTCTCT CGGGTCTGG-3' rev: 5'-GGTGGTTGTGCCGAGAAGGTGGAGA CCGTG-3' at an annealing temperature of 55˚C and 35 cycles.

### Chloroplast isolations

Chloroplasts were isolated from *Arabidopsis* leaves harvested from plants grown under Pi limiting conditions. 10 g Of plant leaves from each plant harvested after Pi induction as described in the Pi induction section (no Pi 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 Pi 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 H2SO4 by placing the leaf material or chloroplasts and H2SO4 in a glass tube and heating over an open flame while stirring, once the leaf material was dissolved completely, 30% (v/v) H2O2 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* lines27 (gift from Scott Poethig, University of Pennsylvania) was transformed with the flower dip method22 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.

## CONCLUSSION:

A high affinity phosphate transporter on the plastid inner envelope is essential for photosynthetic ATP production in phosphate-deprived environments. Phosphorous in the form of orthophosphate (Pi) 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 Pi in soil is extremely low, therefore all plants in their natural environment are always under some degree of Pi deprivation[4,5.] In periods of Pi limitation the Pi concentration in the cytoplasm drops into the low μM range[6,7]. All plastidial Pi transporters characterised to date, however, have a low affinity for Pi and would therefore not function under Pi limiting conditions[2,6,8,9]. Here we show that PHT1;5 is the plastidial transporter responsible for Pi uptake into chloroplasts under Pi-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 Pi is transported in exchange for sugar-phosphates. It is generally accepted that Pi is transported across the chloroplast inner membrane by triose-phosphate/phosphate translocators (TPT), exchanging Pi for phosphorylated carbon intermediates6. The low affinity of these transporters for Pi 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 Pi 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 Pi import into chloroplasts. Without this transporter photosynthesis stops functioning and plants cannot survive in their natural environment. Pi 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 Pi uptake and carbon exchange in chloroplasts, only functions when plants are supplied with sufficient Pi and that another pathway is driving these processes under Pi limitation. Understanding this pathway is an important milestone in understanding how plants are functioning under more sustainable cultivation practises.

When the expression pattern of this gene was investigated under Pi 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 Pi might be imported into chloroplasts through this high-affinity phosphate transporter rather than through TPT under Pi-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 Pi-limitation. To test the contribution of PHT1;5 towards Pi uptake into chloroplasts during periods of Pi limitation, chloroplasts were enriched and the total P content measured in Pi 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 Pi in chloroplasts during Pi limited growth conditions.

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