Phosphate sensing and signalling pathway in Arabidopsis thaliana

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I declare that the work presented in this thesis is my own and that it has not been submitted in any previous application for a degree or qualification. Any contribution made by other parties is clearly acknowledged.
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### Abbreviations

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<tbody>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AHPs</td>
<td><em>Arabidopsis</em> histidine-phosphotransfer proteins</td>
</tr>
<tr>
<td>AM</td>
<td>arbuscular mycorrhiza</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>ARR</td>
<td>auxin response regulator</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>CaMV</td>
<td>Cauliflower Mosaic Virus</td>
</tr>
<tr>
<td>CCD</td>
<td>charged-couple device</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependent kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CHX</td>
<td>cycloheximide</td>
</tr>
<tr>
<td>CK</td>
<td>cytokinin</td>
</tr>
<tr>
<td>Col0</td>
<td>Columbia 0 ecotype (wild type)</td>
</tr>
<tr>
<td>Cyt</td>
<td>cytochrome</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DGDG</td>
<td>digalactosyldiacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FW</td>
<td>fresh weight</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
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<tr>
<td>Glu</td>
<td>glucose</td>
</tr>
<tr>
<td>G3P</td>
<td>glyceraldehyde 3-phosphate</td>
</tr>
<tr>
<td>IAA</td>
<td>indole-3-acetic acid</td>
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<tr>
<td>IAAH</td>
<td>indole acetamide hydrolase</td>
</tr>
<tr>
<td>IPT3</td>
<td>iso-entenyl-transferase</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>KIN</td>
<td>kinetin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LUC</td>
<td>luciferase</td>
</tr>
<tr>
<td>MES</td>
<td>2-morpholinoethanesulfonic acid</td>
</tr>
<tr>
<td>MGDG</td>
<td>monogalactosyl-diacylglycerol</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
</tr>
<tr>
<td>NAM</td>
<td>naphthale acetamide</td>
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<tr>
<td>NR</td>
<td>nitrate reductase</td>
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<tr>
<td>3-OMG</td>
<td>3-o-methyl glucose</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
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<tr>
<td>P</td>
<td>phosphorous</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
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<tr>
<td>PEPC</td>
<td>phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>PFK</td>
<td>phosphofructokinase</td>
</tr>
<tr>
<td>PFP</td>
<td>pyrophosphate dependent phosphofructokinase</td>
</tr>
<tr>
<td>Phi</td>
<td>phosphonate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PPI</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>$^{31}$P-NMR</td>
<td>$^{31}$P nuclear magnetic resonance imaging</td>
</tr>
<tr>
<td>PSI</td>
<td>phosphate starvation induce</td>
</tr>
<tr>
<td>QPCR</td>
<td>quantitative PCR</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RSA</td>
<td>root system architecture</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium aline, sodium citrate</td>
</tr>
<tr>
<td>Suc</td>
<td>sucrose</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>TAIR</td>
<td>the <em>Arabidopsis</em> information resource</td>
</tr>
<tr>
<td>TE</td>
<td>Tris.HCl, ethylene-diaminetetra-acetic acid</td>
</tr>
<tr>
<td>Tween</td>
<td>polyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VAM</td>
<td>vesicular-arbuscular mycorrhiza</td>
</tr>
<tr>
<td>X-gal</td>
<td>4-bromo-4-chloro-3-indol-β-D-galactopyranoside</td>
</tr>
<tr>
<td>YEP</td>
<td>yeast extract peptone</td>
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ABSTRACT

To survive in a phosphate (Pi) deficient environment, plants must adapt by integrating extracellular signals with metabolic status. We analysed immediate-early local and systemic responses to the perception of Pi in Pi-depleted plants or suspension cells. In Pi-depleted plants or cells, RNA abundance of Pi starvation-induced (PSI) genes (the high-affinity phosphate transporter Pht1;1, the monogalactosyl-diacylglycerol synthase MGD3 and At4, a phosphate starvation-induced gene of unknown function) is very rapidly down regulated in response to Pi or phosphonate, a non-metabolic analogue of Pi. This indicates that Pi perception, not its metabolism, is the crucial event in Pi-signalling. We found that PSI gene expression is under negative control by a labile repressor protein or protein complex, which directly controls Pht1;1 and MGD3, but not At4 expression. This labile repressor is regulated by ubiquitin-mediated proteolysis. We also demonstrated that protein kinases are involved early in Pi signalling. Therefore, we proposed a model for local Pi signalling in which the perception of Pi controls the stability of negative regulators of downstream responses. Systemic signalling was examined in a split-root system. Pi perception also initiates systemic Pi signalling. The slow systemic translocation of Pi demonstrates that Pi itself is not the systemic signal. We examined local and systemic signalling in mutant backgrounds affected in PSI responses; only PHO2 significantly affects early Pi signalling.

To assess on the relationship between different nutrient metabolic and signalling pathways, the effects of varying carbon (C) and nitrogen (N) availability on phosphate (Pi) starvation responses and on plant growth were examined. Increased levels of C enhance PSI gene expression responses in roots and shoots, while elevated nitrogen or cytokinin (CK) treatments suppress these responses strongly in roots only. Elevated nitrogen or CK availability shifts shoot-root mass ratios in favour of shoots and alters cellular Pi concentration in both tissues, while increased carbon specifically promotes root growth. Our data indicate that the carbon-nitrogen balance informs growth control networks controlling shoot-root mass ratios. Altered allocation of growth potential by these two nutrients is not dependent on Pi nutritional status and is dominant over Pi starvation-induced growth responses.
To identify genes involved in Pi signalling, a genetic screen with a Pi starvation responsive reporter (luciferase) line was also performed. A candidate mutant was identified and initial analysis showed it is likely to be a signalling mutant.
1 Introduction

1.1 Phosphate and plant

1.1.1 The importance of phosphate

As far as we know, there are more than one hundred and twelve elements in the world. Only a few elements exist in all life forms: phosphorus is one of them. It is an essential element classified as a macronutrient because of the relatively large amounts of P required by plants. The genetic materials DNA and RNA are phosphodiesters. Most of the coenzymes are esters of phosphoric or pyrophosphoric acid. The principal reservoirs of biochemical energy (adenosine triphosphate (ATP), creatine phosphate, and phosphoenolpyruvate) are phosphates (Poirier and Bucher, 2002). Many metabolites are phosphate esters. Phosphate also has multiple functions in growth and development, genetics and evolution, metabolism and signal transduction and numerous other biological processes (Ranjeva and Boudet, 1987). Different with synthetic organic chemistry, in biological reactions phosphate esters or anhydrides are everywhere and can do almost everything (Westheimer, 1987).

Meanwhile in cells, phosphate exists either as inorganic ions or as organic derivatives such as lipid phosphate. Pi is therefore situated at branch points between inorganic and organic metabolism (Mimura, 1999). In various compartments, the concentrations of phosphate in vivo have to be regulated via many cellular activities and phosphate homeostasis is achieved between inorganic ions and organic molecules. To balance phosphate homeostasis is one of the most important mechanisms for all life forms.

Of the major nutrients (N, P and K), phosphorus is the most dilute and the least readily available one in the rhizosphere (Bieleski, 1973; Poirier and Bucher, 2002). In nature, P is widely distributed but not found by itself in elemental form. Elemental P is extremely reactive and will combine with oxygen when exposed to the air. In natural systems like soil and water, P exists as phosphate (Pi), a chemical form in which each P atom is surrounded by 4 oxygen (O) atoms. Orthophosphate, the simplest phosphate, has the chemical formula PO$_4$$^{3-}$. Although the total amount of phosphorous in the soil may be high, the preferred form for assimilation,
orthophosphate (inorganic phosphate), is not easily accessible to most plants and microbes. Inorganic phosphorus in the soil solution normally is precipitated with common cations like calcium (Ca), magnesium (Mg), iron (Fe) and aluminium (Al) phosphate minerals. The increased amount and particle size of calcium carbonates and clay minerals will also increase the precipitation of calcium phosphate minerals on its surfaces. (Rausch and Bucher, 2002). All of these make available Pi concentration in soil for plants to use is really low.

Just because of the poor availability, normally “free” Pi concentrations in the soil solution rarely exceed 10μM (Bieleski, 1973; Marschner, 1995). While in plant cells, the Pi level in the cytoplasm is 1,000-fold higher (1-10mM; (Bieleski, 1973)); for the vacuole where Pi can be stored is even higher than that in the cytoplasm (Mimura, 1999). Therefore the uptake of Pi and maintenance of Pi homeostasis in living cells poses a serious problem to plants.

1.1.2 Plants responses to Pi limitation in soil

Plants absorb huge amounts of phosphate from soil every day. Adequate P availability for plants stimulates early plant growth and hastens maturity. However, many soils throughout the world are Pi-deficient, and even in the more fertile soils, the Pi concentration of the soil solution is still far away from enough (Bieleski, 1973). Corresponding to Pi deficiency in soil, plants have developed numerous morphological, physiological, biochemical and molecular changes to cope with this problem, including changes in root morphology and architecture, accumulation of anthocyanin pigments, secretion of phosphomonoesterases and organic acids into the rhizosphere, improvement of Pi uptake efficiency, and changes in metabolisms. For example, by using cluster or proteoid roots (bottlebrush-like clusters of short rootlets of determinate growth which form along secondary lateral roots) White lupin (Lupinus albus L.) can survive in extremely infertile soil condition and is chosen as a model system for studying phosphorus deficiency (Neumann et al., 2000). Pi stress also results in an increase in anthocyanin accumulation (which may protect leaves from photoinhibition resulting from limited photochemical reactions in photosynthesis (Franco-Zorrilla et al., 2004). More details about how plants adapt to Pi deficiency can be seen in Table 1.1 (Muchhal and Raghothama, 1999).
The ultimate goal of these adaptive changes is to increase Pi availability in the roots, enhance Pi uptake and maintain the basic functions of plant living. Although several of these responses have been reported from a number of agriculturally important plants (Mitsukawa et al., 1997; Yi et al., 2005), still little is known about the underlying molecular processes or regulatory genes involved in the Pi signalling pathways of plants, especially on the early events of Pi sensing and responding pathway(s). After the whole genome has been sequenced, the analysis in Arabidopsis offers the opportunity to get a better understanding of the genetic and molecular background of the Pi signalling pathway. A more complete understanding of Pi uptake and utilisation in plants will not only help people to better understand plants, but eventually have significant implications on the environment and human's life in the world.

Morphological responses
Increased root:shoot ratio; changes in root morphology and architecture; increased root hair proliferation; root hair elongation and density; accumulation of anthocyanin pigments; proteoid root formation; increased association with mycorrhizal fungi

Physiological responses
Enhanced phosphate uptake; reduced phosphate efflux; increased phosphate use efficiency; mobilization of phosphate from the vacuole to cytoplasm; increased translocation of phosphorus within plants; retention of more phosphate in roots; secretion of organic acids; protons and chelaters; secretion of phosphatases and RNases; altered respiration; carbon metabolism; photosynthesis; nitrogen fixation; aromatic enzyme pathways

Biochemical responses
Activation of enzymes; enhanced production of phosphatases, RNases and organic acids; changes in protein phosphorylation; activation of glycolytic bypass pathway

Molecular responses
Activation or adjustment of gene expression (RNases, phosphatases, phosphate transporters, Ca-ATPase, vegetative storage proteins, β-glucosidase, PEPCase, novel genes such as TPSII, Mt 4, At 4

Table 1.1 Multiple responses of plants to phosphate deficiency
(Muchhal and Raghothama, 1999)
1.2 Phosphate in plant

1.2.1 Phosphate uptake from soil to cell

For the plant, Pi uptake starts from soil to the root cells. The form in which Pi exists in soil changes according to pH. The pKs for the dissociation of \( \text{H}_3\text{PO}_4 \) into \( \text{H}_2\text{PO}_4^- \) and then into \( \text{HPO}_4^{2-} \) are 2.1 and 7.2, respectively. In higher plants, Pi uptake rates are highest between pH 5.0 to 6.0 at which \( \text{H}_2\text{PO}_4^- \) is the dominant form of Pi (Ulrich-Eberius et al., 1984; Furihata et al., 1992). Therefore, plants take up Pi in the monovalent form, \( \text{H}_2\text{PO}_4^- \) (Schachtman et al., 1998).

Under normal physiological conditions there is a requirement for energized transport of Pi across the plasma membrane from the soil to the plant because it moves against both electrical and concentration gradients. There are either one or two negative charges taken by Pi molecules in soil and repelled by the negative potential of the cytoplasm (-150 to -200mV) (Mimura, 1999). The concentration of Pi in the cytoplasm is much higher than the Pi concentration in soil. Therefore, the movement of Pi has always to be driven from soil into plant cell.

The driving force for Pi influx is the proton gradient generated by the Pi-type \( \text{H}^+ \)-ATPase (Schachtman et al., 1998; Sze et al., 1999). Plants acquire Pi by an energy-mediated co-transport process with one or more protons (Ullrich-Eberius, 1984). Energised transport from soil to plant is required to overcome the steep concentration gradient between high Pi concentration in plant tissues and low Pi availability in the soil (Schachtman et al., 1998). It has been shown that after addition of Pi, the extracellular pH increased and the cytoplasmic pH first decreased and then maintained a lower value (Sakano, 1990; Sakano et al., 1998). Therefore, an electrochemical proton gradient generated by plasma membrane \( \text{H}^+ \)-ATPases drives this process and absorption of Pi is accompanied by proton influx with a stoichiometry of 2 to 4 \( \text{H}^+ / \text{H}_2\text{PO}_4^- \) (Ullrich-Eberius et al., 1984; Sakano, 1990; Muchhal et al., 1996; Sze et al., 1999). It has been suggested that one of the major differences between plants and animals is the activity of a \( \text{Na}^+ \)-dependent transport system in animal cells (Escoubet et al., 1989; Werner et al., 1998; Werner and Kinne, 2001), whereas \( \text{H}^+ \)-coupled Pi transport system occurs in plants. Dependence of Pi
uptake on Na\textsuperscript{+} has not yet been demonstrated in vascular plants, but the existence of such a transport system still cannot be excluded (Rausch and Bucher, 2002).

Most experimental analyses of Pi transport mechanisms with H\textsuperscript{+} have been proved to be on the high-affinity transporter system. However, analysis of nutrient uptake kinetics into plant roots using a radiotracer medium-depletion method (Drew et al., 1984) revealed that Pi uptake kinetics in plants are generally hyperbolic and monophasic at low Pi concentrations (\(\mu\)M range) in the medium and biphasic when extended to high Pi concentrations (mM range), thus suggesting Pi uptake being mediated by high- and low- affinity transport mechanisms, respectively (Schachtman et al., 1998).

After being taken into rhizodermal cells, there are several destinations for Pi: It may enter the cytoplasmic metabolic pool to be converted into cell components via biochemical pathways; be transported into the vacuole as a form of polyphosphate (polyP) for storage; or be lost via efflux out of the cell. Then, in conditions of adequate Pi, it may be translocated within the whole plant via distinct transport systems (Daram et al., 1999).

### 1.2.2 Phosphate uptake via mychorrizal association

Many plants (> 80% of vascular flowering plants) have the ability to obtain Pi from distant soil areas via symbiotic associations with arbuscular mycorrhizal (AM) fungi, which colonize roots to utilize plant carbon resources (Harrison, 1999). The arbuscular mycorrhiza (AM) is an endosymbiosis in which the fungus inhabits the root cortical cells and obtains carbon provided by the plant whilst transferring mineral nutrients from the soil to the plant cortical cells. Their main contribution is to assist the plant with the acquisition of mineral nutrients, particularly Pi, and recently it was suggested that in an AM symbiosis, plants receive all of their Pi via their fungal symbiont (Harrison, 2005). A unique phosphate transporter, MtPT4, has been identified in Medicago truncatula that is only expressed in arbuscule-containing cells, specifically for the acquisition of Pi released by the fungus (Harrison et al., 2002). Arabidopsis does not form mycorrhizal associations and the majority of research into this form of Pi acquisition is conducted using the model legume, M. truncatula. Therefore Arabidopsis is not a suitable model for mycorrhizae studies.
Pi homeostasis (from the Greek words for “same” and “steady”), that is the processes used to actively maintain a fairly stable Pi condition in the cell, is essential for cell integrity and includes the tight regulation of Pi concentrations and transport into or out of cellular compartments, i.e. the cytosol, plastids, mitochondria, and vacuoles (Mimura, 1999; Poirier and Bucher, 2002). Therefore, in different compartments the Pi concentrations have to be adjusted to achieve Pi homeostasis. For instance, in plant cells the vacuole is a large compartment with a pH of approximately 5 compared to the cytoplasm, which has a pH of approximately 7.5. The Pi signals from these two pools are therefore quite different (Mimura, 1999).

There are many intracellular compartments in plant cells, including nucleus, mitochondria, chloroplasts, golgi apparatus, cytosol, vacuole, etc. Most research on the subcellular distribution of Pi has concentrated on the cytoplasm, the vacuole and plastids (Mimura, 1999). It has been shown that in mesophyll cells, the vacuole occupied about 60-80%, the chloroplasts about 20-30%, and the cytosol 5-10% of total cell volume (Winter et al., 1993, 1994). In vacuolated cells of higher plants the vacuole acts as a storage pool, or ‘non-metabolic pool’, of Pi, and at adequate Pi supply of the plants about 85-95% of the total Pi is located in the vacuole (Bieleski, 1973). In contrast, in leaves of Pi-deficient plants, virtually all Pi is localized to the cytosol and chloroplasts, thus representing the ‘metabolic pool’ of Pi in the plant (Bieleski, 1973; Marschner, 1995). Therefore, vacuolar Pi is used to buffer the cytoplasmic Pi level against fluctuations caused by variable external Pi supply or metabolic activities and to keep Pi homeostasis in the plant cell.

1.2.4 Phosphate translocation within the whole plant

In addition to the uptake system at the root-soil interface, three distinct transport systems are expected to participate in Pi translocation within the plant: (1) secretion of Pi into the xylem in the root; (2) loading of leaf cells with Pi from the vascular system; and (3) cycling within the plant (Daram et al., 1999).

The root tip and the epidermal cells are the most active participants in uptake of Pi into the roots. The flow of Pi transport across the cortex into the xylem occurs over
quite a long time compared with flow through xylem (Bieleski, 1973). It is still difficult to track the pathway of movement from epidermal cells to the xylem. This process should be quite different from transport Pi into the epidermal cells due to the different Pi concentration gradients involved.

Pi is transported from the root to the shoot via the xylem, which is the principal water-conducting tissue of vascular plants. It consists of tracheary elements, tracheids and wood vessels and of additional xylem fibres. All of them are elongated cells with secondary cell walls that lack protoplasts at maturity (Bergfeld et al., 2003). After loading, Pi is released from the xylem to shoot tissues, Pi can be stored in vacuoles of sink cells, redistributed in different organs or transferred back to roots through phloem cells. During leaf senescence or Pi starvation conditions, Pi is redistributed from old source leaves to young sink organs or to the starved roots (Mimura, 1995). Figure 1.1 shows the Pi flow between root cells and shoot cells.

Once inside the cell, Pi passes the membranes of cell organelles, such as plastids (e.g. chloroplasts and amyloplasts), mitochondria, and vacuoles, often exchange with other solutes or protons. Arabidopsis UDP-glucose phosphorylase is induced under Pi starvation and substitutes for ATP-requiring hexose kinase, utilising pyrophosphate (PPI) as an alternative substrate (Ciereszko et al., 2001). Metabolites that are exchanged between the plastids and the cytosol have to be transported across the two envelope membranes. Whereas the outer envelope allows the passage of small molecules, the inner envelope membrane is the actual permeability barrier and the site of Pi and metabolite transport. The solutes to be transported, in exchange with inorganic Pi, are triose phosphates, pentose-phosphates, hexose-phosphates, or phosphoenolpyruvate (PEP). The stoichiometry of these exchanges is important, because it ensures that every molecule of phosphoester transported across either side of the plastid membrane is counterbalanced by the translocation of a Pi molecule to the opposite site (cytosol or plastid) (Poirier and Bucher, 2002).
1.3 Morphological and biochemical responses to Pi limitation

1.3.1 Morphological responses

Low soil Pi availability is a primary constraint to plant growth on earth (Lynch and Deikman, 1998). At the morphological level, the architecture of the root system is modified, with an increase in root/shoot ratio, increase in the length and density of root hairs, as well as proliferation of lateral roots (Lynch, 1995; Bates and Lynch,
Modification of root growth and architecture is a well-documented response to phosphate starvation (Bates and Lynch, 2000a).

Increased root-shoot mass ratio under phosphate starvation is a hallmark of plant responses to phosphate deficiency, enhancing the total surface area available for soil exploration and acquisition of nutrients (Ma et al., 2003). Increased root hair length and density were found to be a result of both increased growth duration and increased growth rate in *Arabidopsis* (Bates and Lynch, 2000b). At 16 days, root hairs of low Pi plants were three times longer compared to root hairs of high phosphate plants (Bates and Lynch, 2000a). Root hair density is about five-time greater in roots grown in low phosphate media compared to high phosphate media. Both root hair length and density were found to decrease logarithmically in response to increasing phosphate media concentration (Bates and Lynch, 2000a). The anatomy of the *Arabidopsis* root is significantly changed under low phosphate media. The root diameter is slightly increased and there are 45% more cortical cells. The number of epidermal cells is also increased while their size is decreased. In *Arabidopsis*, epidermal cells lying over the anticlinal wall separating two cortical cells differentiate into cells that can bear root hairs (trichoblast), whereas those located over the outer periclinal cortical cell walls differentiate into hairless cells (atrichoblast) (Zhang et al., 2003). The large number of cortical cells increases the number of trichoblast files from 8 to 12. The increase in root hair density could be explained by a combination of factors including an increase in the number of trichoblasts, and an increase in the likelihood that a trichoblast will form a root hair, and a greater stacking of hairs from smaller trichoblast (Ma et al., 2003). These results show that phosphate availability can fundamentally affect root cell differentiation and patterning.

Two root morphology mutants (rdh2 and rhd6) have been described (Bates and Lynch, 2000b). The rdh2 mutant is deficient in root hair elongation, the rhd6 mutant shows reduced root hair density. In high Pi (54µM) media, no differences between wild type and the mutants were observed. In contrast, in low Pi (1.5µM) media, wild type plants had greater shoot biomass, total root surface area, absolute growth rate, total Pi content per unit length and specific Pi absorption than the two root hair
mutants, showing the importance of root hairs for Pi assimilation (Bates and Lynch, 2000a, 2000b).

Taken together, plant root systems are highly plastic to adapt their architecture in response to the Pi limitation in soil. Till now, the mechanisms involved in the regulation of root architecture in response to Pi are still unknown. There are several evidence shown that phytohormones such as auxin and ethylene are involved in altering primary root growth, root hair initiation and lateral root formation under different Pi conditions (Torrey, 1976; Lopez-Bucio et al., 2002; Ma et al., 2003). Phosphate deficiency and ethylene may cause similar changes in root systems (Ma et al., 2003). Most of these works are focused on morphological results and need further analysis to show they directly connect with Pi metabolism in plant growth regulation.

1.3.2 Biochemical responses

There are two main functions of biochemical responses to Pi limitation. The first one is increasing the endogenous and soil Pi availability involves increases in Pi uptake capacity through the induction of high affinity Pi transporters, and increasing Pi mobilization and recycling activity through the induction of soil-secreted and endogenous phosphatases and RNases and the increased release of organic acids and protons (Raghothama, 1999; Vance et al., 2003). The induction of acid phosphatases in response to Pi starvation is a universal response in higher plants (Duff et al., 1994). Phosphatases are presumed to liberate Pi from organic materials and its activities have traditionally been used as markers for Pi-deficiency (Baldwin et al., 2001).

The second function is metabolic adaptation to Pi stress, which involves the utilization of alternative glycolytic or respiratory pathways which circumvent metabolic steps requiring Pi or adenylylate, the concentrations of which drop under prolonged Pi stress (Duff et al., 1989). In response to Pi deficiency, many plants show remarkable flexibility in adjusting metabolic rates and utilizing alternative metabolic pathways. The levels of ATP and all of the nucleotides are significantly reduced, whereas pyrophosphate (PPi) is still maintained at a high level (Plaxton, 1996). In the glycolytic pathway, a PPi-dependent phosphofructokinase (PFP) can
‘bypass’ the ATP-dependent phosphofructokinase (PFK) to generate fructose-1,6-biphosphate during Pi starvation condition (Theodorou et al., 1992; Theodorou and Plaxton, 1996). Therefore, PPI has been assumed to be a byproduct of secondary metabolism to be used as a donor to maintain carbon metabolism during Pi starvation (Plaxton, 1996; Plaxton and Carswell, 1999). Pi limitation also results in activation of an alternate respiratory pathway to alternate the cytochrome (Cyt) pathway of electron transport in mitochondria (Rychter et al., 1992; Theodorou and Plaxton, 1993).

Pi stress also results in an increase in anthocyanin accumulation (which may protect leaves from photoinhibition resulting from limited photochemical reactions in photosynthesis; (Takahashi et al., 1991; Trull et al., 1997), in changes carbohydrate metabolism (e.g. increases in starch content), and in thylakoid lipid composition (decreased phosphatidylglycerol may be compensated for an increase in sulpholipids; (Essigmann et al., 1998)).

1.4 Molecular responses to Pi limitation

To accomplish and maintain Pi homeostasis inside a plant, a complex system is needed, including processes of uptake, intracellular partitioning, storage, translocation, and Pi rescue from diverse substrates (Kock et al., 1998). Molecular biological studies in recent years have finally made it possible to identify and investigate the genes that are induced or up-regulated in Pi deficient plants in more detail. Most of the high- and low-affinity Pi transporters in Arabidopsis have been characterised and are summarised below, but several important molecules that are involved in these processes remain unidentified. The framework for Pi signalling is still far from well characterized.

1.4.1 Plant phosphate transporters

Membrane-spanning transport proteins are responsible for the selective passage of most mineral nutrients and metabolites across cellular and intracellular membranes. Transcriptional activation of Pi transporters in response to Pi starvation seems to be a major regulatory mechanism for Pi uptake. Molecular biological studies in recent
years have finally made it possible to identify and investigate high- and low-affinity phosphate transporters in plant.

1.4.1.1 The Pht1 family

The first two Pi transporter genes were isolated from an *Arabidopsis* roots cDNA library (Muchhal et al., 1996). Their protein sequences show high similarity to a high-affinity Pi transporter in yeast and fungus. By expression of these *Arabidopsis* genes in the yeast Pi transporter mutant (*pho84*) the Pi transporter function can be complemented. Since then, a total of seventeen genes from several plant species have been identified as having a high degree of similarity to the yeast high-affinity phosphate transporter, *PH084* (Mudge et al., 2002). These include *Pht1;1, Pht1;2, Pht1;3* and *Pht1;4* genes in *Arabidopsis thaliana* (Muchhal et al., 1996; Smith et al., 1997; Okumura et al., 1998; Dong B., 1999; Mudge et al., 2002); *StPT1, StPT2* and *StPT3* in, *Solanum tuberosum* (potato) (Leggewie et al., 1997; Rausch et al., 2001); *MtPT1* and *MtPT2* in the model legume, *Medicago truncatula* (Liu et al., 1998a; Chiou et al., 2001); and *LePT1* and *LePT2* in *Lycopersicon esculentum* (tomato) (Daram et al., 1998; Liu et al., 1998b; Muchhal and Raghothama, 1999). The structure of Pi transporter proteins is highly conserved between many plant species and fungi. According to computational analyses, their predicted secondary structures contain 12 membrane-spanning domains separated into two groups of six by a large charged hydrophilic region, and a hydrophilic N and C terminus localized in the cytoplasm (Fig. 1.2) (Muchhal et al., 1996; Daram et al., 1998; Poirier and Bucher, 2002).

An *Arabidopsis* Information Resource (TAIR) Gene Search at [http://arabidopsis.org/info/genefamily/genefamily.html](http://arabidopsis.org/info/genefamily/genefamily.html) reveals the existence of nine genes in the haploid *Arabidopsis* genome coding for inorganic Pi transporters that exhibit high sequence similarity to each other. In accordance with the rules recommended by the Commission on Plant Gene Nomenclature ([http://mbclserver.rutgers.edu/CPGN/](http://mbclserver.rutgers.edu/CPGN/)) this plant gene family was named the *Pht1* family (Bucher et al., 2001). A phylogenetic tree of the nine *Arabidopsis* *Pht1* members is shown in figure 1.3. Interestingly, *Pht1;1, Pht1;2, Pht1;3* and *Pht1;6* are clustered within 24,200bp on
chromosome 5, suggesting the occurrence of gene duplication events during the evolution of *Pht1* gene family (Poirier and Bucher, 2002).

![Fig. 1.2. Predicted topology of the tomato *Pht1;1* (*LePT1*) Pi transporter (Daram et al., 1998) typical for *Pht1;1* proteins, with 12 transmembrane helices, a cytoplasmic N and C terminus and a long cytoplasmic loop between transmembrane helices 6 and 7. Numbers indicate amino acids with the start methionine marked as number 1.](image)

At least eight members of the *Arabidopsis Pht1* gene family are expressed in roots (Mudge et al., 2002). Under Pi-depleted conditions, four of these genes (genes promoter drives reporter gene) were found to direct expression in the root epidermis (Mudge et al., 2002). It suggests this gene family plays multiple roles in phosphate uptake and remobilisation. *Pht1;2* and *Pht1;3* have spatial expression patterns that overlap partially with *Pht1;1* and *Pht1;4*. *Pht1;1* and *Pht1;4* were also demonstrated to play a major role in Pi acquisition as low- and high-affinity Pi transporters (Shin et al., 2004). *Pht1;1* and *Pht1;4* were able to complement the yeast *pho84* high-affinity Pi-transporter mutant (Muchhal et al., 1996). *Pht1;1* over-expression was found to enhance Pi uptake into tobacco cells (Mitsukawa et al., 1997). In addition, the
double mutant shows a 75% reduction in Pi uptake capacity relative to wild type under high-Pi condition (Shin et al., 2004).

Moreover, the expression data shows that Pht1;1 is expressed mainly in root hairs and emerging secondary roots, with strong expression in epidermal and endodermal layers. Pht1;2 gene expression was observed in all cell types of the root meristematic region and in the epidermis, cortex and stellar region of the mature root (Karthikeyan et al., 2002). Pht1;3 expression was found in the stele and pericycle cell layer, whereas Pht1;4 expression was observed in the stele, root tips and epidermis. Furthermore, the strongest Pht1;1, Pht1;2 and Pht1;3 expression was located in the root hair-forming trichoblast cells (Mudge et al., 2002). These expression patterns indicate this gene family plays an important role in Pi uptake and also emphasize the significance of root hairs in Pi acquisition (detail see table 1.2).

1.4.1.2 Other phosphate transporter gene families

Following the sequencing of *Arabidopsis* genome, the Pht2;1 DNA was reported to share similarity with bacterial and eukaryotic Pi transporters (Daram et al., 1999). It's fairly high apparent Km for Pi (0.4 mM) and high mRNA abundance around the vascular tissue in leaves, based on RNA *in situ* hybridization studies, suggested a role in Pi loading of shoot organs (Daram et al., 1999). Although the protein
sequence is highly similar to that of eukaryotic sodium-dependent Pi transporters, functional analysis of the *Pht2;1* protein in mutant yeast cells indicated that it is a H+/Pi symporter. The independent efforts of three groups subsequently showed that *Pht2;1* is localized to the chloroplast (Versaw and Harrison, 2002). Fusions of the entire *Arabidopsis Pht2;1* protein or the N-terminal part of the transporter to GFP indicated transport of the fusion proteins to the chloroplast (Poirier and Bucher, 2002; Versaw and Harrison, 2002). A subcellular proteomics approach, which was developed to identify the most hydrophobic chloroplast membrane proteins in spinach, in combination with immunolocalization and GFP fusion studies identify the spinach *Pht2;1* protein as an integral protein of the inner envelope membrane of chloroplasts (Ferro et al., 2002).

The envelope membrane of organelles contains specific translocators that are involved in transport processes of different intermediates. *Arabidopsis Pht3* gene encode a small family of mitochondrial Pi transporters and show homology to the first plant mitochondrial Pi translocator cDNA (*Mptl*) cloned from birch (*Betula pendula Roth*) in a screening for ozone-inducible genes (Kiiskinen et al., 1997).

Recently, Phosphate transporter traffic facilitator1 (*PHF1*) was reported to impair localization of Pi transporter(s) and result in the constitutive expression of many Pi starvation-induced genes (Gonzalez et al., 2005). It was specifically responsive to Pi starvation and was localized in endoplasmic reticulum (ER). The function of this protein is to target phosphate transporters to the plasma membrane in *Arabidopsis* (Eckardt, 2005).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene IDx</th>
<th>Published name(s)</th>
<th>Acc.nos.</th>
<th>SwissProt</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pht1;1</td>
<td>At5g43350</td>
<td>PHT1&lt;sup&gt;5&lt;/sup&gt;, APT2&lt;sup&gt;5&lt;/sup&gt;, AtPT1&lt;sup&gt;9&lt;/sup&gt;</td>
<td>D86591,D86608, Y07692, U62330</td>
<td>Q96302, Q96264, Q96302</td>
<td>Root, cotyledon&lt;sup&gt;f&lt;/sup&gt;, shoot&lt;sup&gt;bud&lt;/sup&gt;, seed&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht1;2</td>
<td>At5g43370</td>
<td>PHT2&lt;sup&gt;2&lt;/sup&gt;, APT1&lt;sup&gt;2&lt;/sup&gt;</td>
<td>AB000094, Y07681</td>
<td>Q48640, Q96243</td>
<td>Root</td>
</tr>
<tr>
<td>Pht1;3</td>
<td>At5g43360</td>
<td>PHT3&lt;sup&gt;3&lt;/sup&gt;, AtPT4&lt;sup&gt;4&lt;/sup&gt;</td>
<td>AB000094, U97546</td>
<td>O48639, O04381</td>
<td>Root&lt;sup&gt;f&lt;/sup&gt;, cotyledon&lt;sup&gt;f&lt;/sup&gt;, leaf&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht1;4</td>
<td>At2g38940</td>
<td>PHT4&lt;sup&gt;4&lt;/sup&gt;,</td>
<td>AB016166</td>
<td>Q96303</td>
<td>Root, leaf, suspension</td>
</tr>
</tbody>
</table>

23
Table 1.2 List of members of Arabidopsis thaliana Pi transporter families. Tissue localization by Northern blots. (Poirier and Bucher, 2002)

<table>
<thead>
<tr>
<th>Pht;1:5</th>
<th>At2g32830</th>
<th>PHT5&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AC003974</th>
<th>O50040</th>
<th>Leaf&lt;sup&gt;f&lt;/sup&gt;, flower bud&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AB000093</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AC003033</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pht;1:6</td>
<td>At5g43340</td>
<td>PHT6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>AB005746</td>
<td>Q9ZWT3</td>
<td>Cotyledon&lt;sup&gt;f&lt;/sup&gt;, pollen&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht;1:7</td>
<td>At3g54770</td>
<td>-</td>
<td>ATT5N23</td>
<td>T47629&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Root&lt;sup&gt;f&lt;/sup&gt;, flower&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht;1:8</td>
<td>At1g20860</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Root&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht;1:9</td>
<td>At1g76430</td>
<td>-</td>
<td>AAF20242</td>
<td>-</td>
<td>Root&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pht2;1</td>
<td>At3g26570</td>
<td>-</td>
<td>AJ302645</td>
<td>Q38954</td>
<td>Aerial organs</td>
</tr>
<tr>
<td>Pht3;1</td>
<td>At5g14040</td>
<td>-</td>
<td>BAB08283</td>
<td>Q9FMU6</td>
<td></td>
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<tr>
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<td>-</td>
<td>-</td>
<td>T49281&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Pht3;3</td>
<td>At2g17270</td>
<td>-</td>
<td>-</td>
<td>B84550&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

1.4.2 Genes are involved in phosphate signalling and metabolism

Many genes involved in Pi-starvation responses have been cloned and their functions demonstrated in *Arabidopsis*, as in bacteria and yeast. However, whilst in bacteria and yeast the mechanisms underlying the control of Pi-starvation responses are quite clear, in *Arabidopsis*, this regulatory system is just beginning to be elucidated.

1.4.2.1 *PHO1*

The first genetic screen for mutants affected in phosphate acquisition was done by Poirier (Poirier et al., 1991). After screening 2200 EMS-mutagenized *Arabidopsis* plants, a mutant deficient in the accumulation of inorganic Pi in leaves was isolated (Poirier et al., 1991). The mutant, named *pho1*, shows classical phenotypes of Pi
deficiency, including reduced growth and accumulation of anthocyanins. It accumulates approximately 5% inorganic Pi and 24% to 44% total Pi in leaves relative to Wt., while Pi in roots is similar to wild type (Poirier et al., 1991; Yves Poirier, 1991; Delhaize and Randall, 1995). The results indicated that phol mutant was impaired in a protein involved in the loading of Pi into the xylem of roots in Arabidopsis.

The PHO1 gene was identified by a mapping based positional cloning strategy. It was found to be mainly expressed in the roots and only weakly up-regulated by Pi deprivation stress (Hamburger et al., 2002). Promoter-GUS fusion experiments revealed predominant expression of the PHO1 promoter in the stellar cells of the roots and the lower section of the hypocotyls (Wang et al., 2004b). This pattern is consistent with the role of PHO1 in loading Pi out of the stellar cells for its transport into the xylem (Poirier et al., 1991). Interestingly, the Arabidopsis genome contains 10 additional genes showing homology to PHO1 (Hamburger et al., 2002). All members in this family have the same topology and harbor a SPX tripartite domain in the N-terminal hydrophilic portion and an EXS domain in the C-terminal hydrophobic portion (Wang et al., 2004b). The SPX domain was named after the proteins SYG1 and PH081 of yeast and XPR1 of human. It can be subdivided into three smaller domains of 35 to 47 amino acids and in most case, these three subdomains are found close together. The EXS domain was named after the yeast ERD1, involved in the localization of endogenous endoplasmic reticulum proteins, the human XPR1, and the yeast SYG1. In Arabidopsis, only the members of the PHO1 proteins family possess both a SPX and EXS domain. These domains were identified from yeast, their function was likely involved in Pi transporter or sensing or in sorting proteins to endomembranes (Wang et al., 2004b). Their expression patterns indicates a likely role of the PHO1 proteins not only in the transfer of Pi to the vascular cylinder of various tissues but also in the acquisition of Pi into cells, such as pollen or root epidermal/cortical cells, and in maintaining Pi homeostasis in the plant (Poirier and Bucher, 2002; Wang Y, 2004).

1.4.2.2 PHO2
Based on a screen using X-ray fluorescence spectrometry (XRFS) analysis (Delhaize, 1993), Delhaize and Randall have characterized the second Pi mutant, pho2. Pho2 mutants accumulated up to 3-fold more total P in leaves, mostly as inorganic phosphate (Pi), than wild-type seedlings. Compared to wild-type plants, pho2 mutants had greater Pi concentrations in stems, siliques, and seeds, but roots of pho2 mutants had similar or lower Pi concentrations than either phol mutants or wild-type seedlings. Under high transpiration conditions, pho2 leaves showed Pi toxicity symptoms - the whole leaf senesced (Delhaize and Randall, 1995). Although toxicity symptoms were influenced by transpiration rates, the higher Pi level in leaves of pho2 were observed even in plants grown submerged in liquid solution, indicating that the higher accumulation of Pi in leaves was independent of transpiration (Poirier and Bucher, 2002). Further more, comparison of the uptake and translocation of Pi by pho2 with that of wild-type plants showed that pho2 plants had about a two-fold greater Pi uptake rate than wild-type plants under P-sufficient conditions and a greater proportion of the Pi taken up accumulated in shoots of pho2 plants (Dong et al., 1998).

Recently, by a positional cloning approach, the PH02 gene has been identified and its identity was confirmed by molecular functional complementation (Bari et al., 2003). PHO2 encodes an ubiquitin-conjugating enzyme E2-like protein, which is involved in selective protein degradation via the 26S proteasome (Aung et al., 2006). The conjugating E2 enzyme actually catalyzes the attachment of ubiquitin to the substrate protein. Normally, the proteasome is the structure that actually does the degradation. Degradation is not the only fate possible for ubiquitin-tagged proteins; ubiquitination also regulates certain processes by mechanisms that do not appear to involve proteolysis, including ribosomal function, post-replicational DNA repair, the initiation of the inflammatory response and the function of certain transcription factors (Pickart, 2001). The PHO2 gene expression pattern has shown its expression in all major plant organs, including roots, leaves, stems, flowers and siliques. Using promoter-GUS fusions revealed a predominant expression in the vascular tissues of roots, leaves, stems, and flowers (Bari et al., 2003). The PHO2 gene has been suspected to be involved in phloem transport of Pi between shoots and roots or in regulating leaf Pi concentration (Dong et al., 1998).
1.4.2.3 pho3

The pho3 mutant was isolated by screening for root acid phosphatase (APase) activity in plants grown under low-Pi conditions. pho3 had 30% less APase activity in roots than the wild type and, in contrast to wild-type plants, root APase activity did not increase in response to growth in low Pi. However, shoot APase activity was higher in pho3 mutant than in the wild-type plants. In addition, the pho3 mutant had a P-deficient phenotype, even when grown in P-sufficient conditions. The results suggest that the mutant is unable to respond to low internal Pi levels, and may lack a transporter or a signaling component involved in regulating Pi nutrition (Zakhleniuk et al., 2001).

1.4.2.4 AtIPS1 & At4

AtIPS1&At4 genes (genes induced by phosphate starvation) are the orthologous genes in Arabidopsis which belong to TPSII/Mt4 gene family (Burleigh and Harrison, 1997). TPSII was isolated from tomato (Liu et al., 1997). Mt4 was isolated as a differential screen to identify genes showing altered expression during the interaction between Medicago truncatula and the vesicular-arbuscular mycorrhizal (VAM) fungus Glomus versiforme (Burleigh and Harrison, 1997). This gene is the first gene to be identified whose expression is altered independently by both mycorrhizal colonization and Pi nutrition.

The members of this gene family encode short, non-conserved open reading frames, respond rapidly and specifically to P deficiency (Martin et al., 2000). Their 5'-flanking regions encode a conserved cis-element, similar to those (CACG TG/T) essential for the expression of Pi starvation induced genes belonging to the pho-regulon of yeast, detail shown in section 1.5 (Ogawa et al., 1995a). In yeast, the promoters of Pi-regulated genes (PHO5, PHO11, PHO8, PHO81, PHO84) contain 1 to 5 copies of this conserved sequence. The PHO4 gene product, a trans-acting factor, specifically interacts with CACG T(G/T) motifs with varying affinities (Ogawa et al., 1995a). These genes represented a novel type of P-responsive gene thought to be involved in regulating plant responses to P deficiency (Burleigh and Harrison, 1997; Liu et al., 1997) and are likely to function as ribosome regulators controlling the function of other molecules such as RNA, DNA or proteins.
1.4.2.5 PHRJ

PHRJ (phosphate starvation response 1) was identified in a EMS-mutagenized genetic screen for mutants in the Pi-starvation response in Arabidopsis, using a line harbouring the highly specific AtIPS1::GUS reporter (Rubio et al., 2001). Phr1 mutants displayed reduced responses of most Pi-starvation responsive genes to Pi starvation (Poirier and Bucher, 2002). In addition, phrl plants showed reduced anthocyanin accumulation in response to Pi starvation and, to a lesser extent, reduced root-to-shoot growth ratio, indicating that PHRJ is a positive regulator of the Pi-starvation response (Rubio et al., 2001). An interesting observation concerning the effect of phrl on anthocyanin accumulation is that this effect is specific for Pi starvation, because accumulation of this pigment following other stimuli, such as nitrogen starvation, cytokinin or ABA treatment was unaffected in this mutant (Rubio et al., 2001). This indicates that responses shared between different stresses may be controlled by at least partially independent regulatory systems (Franco-Zorrilla et al., 2004).

PHRJ was positionally cloned and found to encode a MYB transcription factor with homology to PSR1 (Phosphorous starvation response 1) from Chlamydomonas reinhardtii (Wykoff et al., 1999; Rubio et al., 2001). PHRJ and PSR1 share, in addition to a MYB DNA binding domain, a second domain predicted to form a coiled coil (CC), a fold usually involved in protein–protein interactions. Thus, the MYB domain was likely to be involved in sequence-specific recognition, and the coiled-coil domain may be the dimerization domain (Rubio et al., 2001). PHRJ, itself, is not highly Pi-responsive and is located in the nucleus independent of the Pi status of the plant, indicating that either PHRJ activity is regulated post-transcriptionally or that a second Pi-starvation regulatory protein is also needed to mount a proper Pi-starvation response.

1.4.2.6 Other Pi responsive genes

Following by the complete sequencing of the Arabidopsis genome and involvement of new technologies in plant Pi-starvation responses, including micro-array and AffimmetrixTM, more and more mutants and genes were isolated for their responses to Pi starvation. AtACP5 was found to be related to purple acid phosphatases (Del Pozo
et al., 1999). SIZI is a plant small ubiquitin-like modifier (SUMO) E3 ligase and a focal controller of Pi starvation-dependent responses (Miura et al., 2005). PDR2 disrupts the local Pi sensing by selective inhibition of root cell division (Ticconi et al., 2004). The ars1 mutant showed increased tolerance to arsenate (a structure analogue of Pi) and increased Pi uptake (Lee et al., 2003). Genes involved in the subsequent utilization of DAG to synthesize galacto-lipids (MGDG, DGDG) were strongly up-regulated at the early stages of Pi deprivation (Misson et al., 2005). One of the miRNA (miR399) gene families was also involved in Pi starvation (Jones-Rhoades and Bartel, 2004). Taken together these classical genetics approaches with all the tools of functional genomics should allow us to gain new insight into the network of the Pi signaling pathway.

1.4.3 Transcriptional control of Pi limitation

Although there has been an increasing amount of molecular data about the genes that are induced or up-regulated in Pi-deficient plants in recent years, still little is known about the molecular mechanisms underlying Pi sensing in plants. The nature of the Pi-sensing system at the cellular level was characterized by Köck (1998), using tomato cell culture to compare the intracellular and extracellular RNases (Pi-starvation induced genes) RNA abundance. Under +Pi conditions, when cells were incubated with D-mannose and other metabolites, which are known to sequester intracellular Pi into organic compounds, the Pi-starvation responsive RNase genes were rapidly induced. These results indicate that Pi sensing itself is intracellular (Kock et al., 1998).

At the whole plant level, there are two types of responses: one dependent on whole plant Pi status involving long-distance (systemic) signalling and the other dependent on the changes of external Pi concentration involving local signalling (Franco-Zorrilla et al., 2004). Using a split-root technique in which the roots of Pi-depleted plants were divided and one part was exposed to a high Pi medium, while the other was still in a low Pi medium, both of the local and systemic signalling pathways are initiated respectively (Burleigh and Harrison, 1999). In the split root system, Pi-starvation responses are systemically repressed in the parts of the roots exposed to low Pi medium. Also, because of the absence of Pi accumulation in the Pi-deplete
roots, the systemic signal does not appear to be Pi itself (Burleigh and Harrison, 1999). Taken with the observations of the pho1 mutant, these results suggest that the systemic signal has a repressor function and originates from the +Pi parts of the plant, rather than an activation function originating from Pi-depleted shoots (Franco-Zorrilla et al., 2004). Pi starvation-responsive genes appear to be involved in multiple metabolic pathways, indicating a complex Pi regulation system in plants (Wu et al., 2003).

1.5 Phosphate signalling in bacteria and yeast

In response to low phosphate availability, microorganisms have evolved a multi-gene emergency rescue system to scavenge traces of usable phosphorus from the environment. Increased production of secreted phosphatases and of high-affinity phosphate-transporters is a common first response. The mechanisms that control the adaptation of *Escherichia coli* (Vershinina and Znamenskaia, 2002) and *Saccharomyces cerevisiae* (Lenburg and O'Shea, 1996) to phosphate limitation have been extensively studied and numerous components of the regulatory networks linking phosphate levels to gene activation have been described. At least 30 genes are co-ordinately de-repressed by phosphate starvation and are likely to play a role in phosphate assimilation (Oshima, 1997).

The two-component system includes a histidine kinase protein that receives a signal and transmits it to a partner response regulator protein. The response regulator protein in turn transmits the signal to the target. The signal is transmitted between the histidine kinase and the response regulator via phosphorelay. Specifically, the histidine kinase autophosphorylates (using ATP as the phosphoryl donor) at a histidine residue in the carboxyl-terminal region (comprising approximately 240 amino acids) called the transmitter domain in response to a stimulus, and then transfers the phosphoryl group to an aspartate residue in the amino-terminal region (comprising about 120 amino acids) of the partner response regulator protein called the receiver domain. This activates the response regulator, which transmits the signal to its target.

In *E. coli*, a two-component signalling system is encoded by the *phoB-phoR* operon. *PhoR* is a histidine protein kinase located in the inner membrane and acts as a Pi

In *S. cerevisiae*, this increase in Pi uptake, as well as many other Pi starvation responses, is regulated by the Pi signal transduction pathway, the *PHO* pathway (Ogawa et al., 2000). The *PHO* pathway (Lenburg and O'Shea, 1996; Oshima, 1997) in budding yeast has been genetically defined by assaying for the activity of *Pho5* protein, a broad specificity acid phosphatase, whose transcription is induced in response to Pi starvation. Central to the *PHO* pathway is a cyclin/cyclin-dependent kinase complex (*CDK; Pho80/Pho85* protein complex) whose activity is regulated in response to external Pi concentrations (Kaffman, 1994). *Pho81* protein, a *CDK* inhibitor, binds to the cyclin-*CDK* when cells are grown in both high and low Pi conditions but only markedly inhibits the kinase in vivo during Pi starvation (Schneider, 1994; Ogawa et al., 1995b). Mutations that inactivate the cyclin-*CDK* lead to constitutive expression of *PHO5*, and null mutations in the *CDK* inhibitor result in an inability to induce *PHO5* (Lemire et al., 1985) Fig 1.4.

*Pho4* is the transcription factor that binds a consensus nucleotide (*Pho4* box) found in the promoters of genes forming the regulon, which includes the Pi transporters *Pho84* and *Pho89* as well as the acid phosphatase *Pho5*. The level of phosphorylation controls activation of *Pho4*. Whereas the hyper-phosphorylated *Pho4* is mainly located in the cytoplasm, the hypo-phosphorylated form is found predominantly in the nucleus where it can act to activate transcription. Under low Pi conditions, the *Pho81* protein inhibits the *Pho80-Pho85* complex, preventing phosphorylation of *Pho4*, and thus activating transcription of the regulon. Alternatively, under high Pi availability, the *Pho80-Pho85* kinase phosphorylates
Pho4. Pho4 interacts with Pho2 which itself activates transcription of only a subset of Pho4-dependent genes.

Interestingly, the Pho85 cyclin-dependent kinase is also involved in several signal transduction pathways in yeast. As well as Pi signaling pathway, the responses mediated by Pho85 include cell-cycle progression and carbon metabolism. Although these responses require the phosphorylation of different substrates, and have different mechanistic consequences as a result of this phosphorylation, all appear to be involved in responses to changes in environmental conditions and to inform the cell that the current environment is satisfactory (Adam and O'Shea, 2002).

It is possible that a phosphate regulon such as that found in yeast may also operate in plants. The induction of acid phosphatases and high-affinity phosphate transporters
under phosphate deprivation are responses that are conserved between the two organisms. In fact, genes that encode high-affinity Pi transporters in plants were originally isolated due to their homology to the yeast PHO84 gene. However, signalling and regulation of responses will ultimately be more complex in multicellular organisms because different organs, tissues and cells will experience varying Pi levels at any one time. Different signalling pathways may therefore operate at the local and systemic level.

1.6 Phosphate and cytokinin signalling

Cytokinins were first identified as factors that promote cell proliferation and sustained growth in cultured plant cells (Miller et al., 1955; Miller et al., 1956). They influence many aspects of growth and development, including seed germination, vascular development, cell proliferation, apical dominance and leaf senescence (Fernando and Kieber, 2005). The current model for cytokinin signalling is a His-Asp multi-step phosphorelay cascade similar to bacterial two-compartment systems that are used in responses to environmental stimuli (Hwang and Sheen, 2001; Heyl and Schmulling, 2003). This two-component system comprises histidine protein kinases that sense the input, histidine phosphotransfer proteins and response regulators that mediate the output to control signal transduction pathways.

In Arabidopsis cytokinin receptor kinases (AHK2, AHK3, AHK4/CRE1/WOL) contain a conserved extracellular cytokinin-binding domain, a histidine kinase domain and a receiver domain. The five Arabidopsis histidine-phosphotransfer proteins (AHPs) mediate the phospho-transfer from the receptor kinases to the response regulators. The 23 response regulators (ARR) genes fall into two main groups on the basis of their sequence similarities, domain structure and transcriptional response to cytokinin (D'Agostino et al., 2000; Mason et al., 2004). In Arabidopsis type-A ARRs with only the receiver domain, might be negative regulators of cytokinin responses and type-B ARRs with the receiver domain fused to the DNA-binding domain, are transcription factors that localize to the nucleus (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2004; To et al., 2004) and can bind with the promoters of many of the cytokinin primary response genes.
Several type-B *ARRs* have been shown to be positive elements in cytokinin signaling (Hwang and Sheen, 2001; Sakai et al., 2001).

There is increasing evidence that cytokinin might relay the nutritional status of the plant. Supplying nitrogen to nitrogen-starved plants leads to increases in cytokinin transport from the roots. Cytokinin is translocated via the xylem to the leaves, where it leads to the accumulation of type-A response regulators (Takei et al., 2001; Takei et al., 2002). Cytokinins block the induction of several genes that are up regulated in response to phosphate starvation. This repression is mediated through *CRE1* (Franco-Zorrilla et al., 2002). Interestingly, the expression of *CRE1* itself is down regulated by phosphate starvation, suggesting a positive feedback loop. In *Arabidopsis* roots, *CRE1* was also shown to negative regulate sulfate uptake in sulfate-replete conditions (Maruyama-Nakashita et al., 2004). Together, these results suggest that changing cytokinin levels and/or changing sensitivities might play a key role in modulating the balance between shoot and root growth in response to altering carbon/nutrient ratios (Fernando and Kieber, 2005).

### 1.7 Genetic screen and phosphate signalling

"Molecular genetic studies rely on well-characterized organisms that can be easily manipulated. *Arabidopsis thaliana* (the model system of choice for plant biologists) allows efficient analysis of plant function, combining classical genetics with molecular biology. Although the complete sequence of the *Arabidopsis* genome allows the rapid discovery of the molecular basis of a characterized mutant, functional characterization of the *Arabidopsis* genome depends on well-designed forward genetic screens, which remain a powerful strategy to identify genes that are involved in many aspects of the plant life cycle." (Page and Grossniklaus, 2002).

As previous section shows, scientists have already identified quite a few mutants of which are involved in Pi metabolism by using this approach and it has already been proved to be a successful method to dissect Pi signalling pathway. The success of a forward genetic screen depends mainly on two factors: first, the definition of a suitable genetic background; and second, an easy and tight procedure to identify the mutants of interest (Page and Grossniklaus, 2002).
In the classic genetic screen, there are two ways to perform mutagenesis in *Arabidopsis*, the insertional mutagenesis and the chemical or physical mutagenesis. The insertional mutagenesis approach is based on the *Arabidopsis* can easily be transformed by *Agrobacterium*-mediated gene transformation; this transfer can randomly integrate a DNA sequence, which is a part of a large bacterial plasmid, into the plant nuclear genome. Based on the highly efficient T-DNA transformation protocol, a single treatment can generates thousands of independent transformants. Recessive mutants are not observed in the progeny from the transformed plant (T1), but rather in the subsequent generation (T2). The main drawbacks of T-DNA mutagenesis are the often complex integration patterns of T-DNA, including transfer of vector sequences that flank the T-DNA, multiple insertions and the high frequency of concatemeric insertions, which can complicate the identification of flanking sequences (De Buck et al., 1999).

Transposons were discovered first by McClintock who first described the maize Activator-Dissociation (*Ac-Ds*) transposable element system (McClintock, 1948). In the *Ac-Ds* transposon mutagenesis system, the two starter lines are transgenic plants that carry T-DNA that contains the Ac and the Ds element, generating genetically mosaic F₁ plants. Ds elements preferentially transpose to sites that are closely linked to the donor site, so the starter lines were designed that allow positive selection for the presence of a newly transposed Ds element and negative selection against the Ac and the Ds donor locus. The immobilized Ac transposase source in the F₁ is counter-selected in the F₂. The Ac T-DNA vector contains an indole acetamide hydrolase (IAAH) gene that confers sensitivity to naphthale acetamide (NAM). Ds, which carries the *NPTII* gene that confers kanamycin resistance, can be positively selected using kanamycin. To select against Ds elements linked to the donor Ds, the donor Ds T-DNA construct also contains the counter-selectable marker gene *IAAH*. This combination selects against Ac transposase, the donor Ds and Ds insertions that are linked to it, whilst selecting for unlinked Ds elements. The new transposon lines are maintained by self-pollination and are screened for phenotypes, or reporter gene expression patterns (Long et al., 1993; Sundaresan et al., 1995).

In the physical mutagenesis, the most commonly methods are ionizing radiation, such as γ and X-rays, or fast neutrons. Ethyl-methane sulfonate (EMS) leads to base
pair substitutions, like GC/AT, and is often used in chemical mutagenesis in Arabidopsis. All of these mutagenesis leads to a loss of function recessive mutant. So the mutant phenotype cannot be observed in the plant in which the mutations occurred. The next generation from which their parents have been mutagenized is called the M1 generation and is heterozygous for the mutations. The plants that grow out from the seeds formed by M1 plants are M2 generation. In this generation, the homozygous recessive mutations will segregate and can be detected by screen.

To identify interesting mutants by genetic screen, the simpler the screening procedure is operated, the larger amount of plants can be screened. For example, to identify the cytokinin signal mutants, hypocotyl segments of M2 seedlings were aseptically excised and cultured on the media with cytokinins. Normal calli showed rapid growth and turned to green without forming root primordial. The mutant candidates which reduced green colour with root primordia were shown to have lost responses to cytokinins (Kakimoto, 1996; Inoue et al., 2001). By using this approach, around 20,000 to 50,000 seedlings were screened and several important elements were identified including cytokinin receptor in Arabidopsis (Inoue et al., 2001).

To identify mutants of which do not have phenotypes or have phenotypes that are difficult to detect, the reporter genes can be used to engineer a highly specific background to dissect virtually any process of interests. Normally, a reporter gene encoding firefly luciferase (LUC), β-glucuronidase (GUS) or green fluorescent protein (GFP) is fused to the promoter of a specifically regulated or inducible gene. The wild type expression pattern of the reporter gene can be observed. In the candidate mutants, a deviation from the wild-type expression pattern can be detected after mutagenesis (for example, see (Millar et al., 1995)). Based on different purposes and targets, several different modified screen approaches also have been developed, like second-site modifiers to further screen for second-site mutations, which either enhance or suppress the primary phenotype (Forsburg, 2001).

Luciferase is a generic name for enzymes commonly used in nature for bioluminescence. The name itself is derived from Lucifer, which means light-bearer. The most famous one is firefly luciferase (EC 1.13.12.7) from the firefly Photinus.
In luminescent reactions, light is produced by the oxidation of a luciferin (a pigment), sometimes involving Adenosine triphosphate (ATP). The reaction takes place in two steps:

\[
\text{luciferin} + \text{ATP} \rightarrow \text{luciferyl adenylate} + \text{PPi}
\]

\[
\text{luciferyl adenylate} + \text{O}_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light}
\]

Normally, luciferase is used as a reporter to analyze specific gene expression mechanism in vivo. In my studies, the Pi responsive genes promoter fused with luciferase gene were transformed in Arabidopsis to generate a homozygous reporter line, as described in Chapter 5. Based on this reporter line, after mutagenesis, any mutant of which affects the Pi responsive genes expression in plants can be identified by the images of bioluminescence (detail in Chapter 5).

After the mutant has been identified, map-based cloning approach is used to seek the genes underlying this mutant. If a mutation is caused by the insertion of a T-DNA or transposon, the inserted sequence provides a tag pointing directly to the gene. However, chemical agents and radiation mutagenesis are not this kind. By using the sequence of the genome and high-resolution markers in Arabidopsis, the positional cloning approach becomes simple, reliable and inexpensive.

In phosphate signalling pathway in Arabidopsis, although there are several genes and proteins have been implicated in Pi transport and homeostasis, there still lack information to deeply characterize on the whole of this important process. By using genetic screen approach, it will help us to identify new components to understand the Pi transport across all membranes, as well as on the Pi regulation and homeostasis.
2 Materials and Methods

2.1 Arabidopsis plant and cell growth methods

2.1.1 Seed sterilisation

Ethanol method:

- Aliquot Arabidopsis seeds in 1.5 ml Eppendorf tubes;
- Pipette 1 ml of 70% ethanol with Tween 20 (20 µl Tween in 100 ml ethanol) for 10~15 min with periodic shaking, then remove the ethanol;
- Wash the seeds with 95% ethanol 3~5 min twice;
- Spread out the autoclaved filter papers in the sterile hood and squirt the seeds on the filter papers;
- Let the seeds dry completely and then put them into eppendorf tubes with 0.1% agarose and keep in the cold room.

Bleach method:

- Aliquot Arabidopsis seeds in 1.5 ml Eppendorf tubes;
- Well separate the tubes onto rack with the lids opened and mark the tubes with lead pencil and put the rack in a jar;
- Put a 250 ml beaker into the same jar and add 100 ml bleach. Then put the whole setup into the fume hood;
- Pipette 3~5 ml concentrated HCl into the beaker and close the lid of the jar immediately;
- After 4~12 hours, sit the opened tubes in the sterile hood to allow the chlorine gas diffused away;
- Pipette 1 mL 0.1% agarose into the tubes and close the lids. The seeds are kept into the cold room waiting for planting.
2.1.2 Plant growth media

Arabidopsis seedlings are grown in half strength MS media (Murashige and Skoog, 1962) or half strength Johnson media (Johnson et al., 1957). Plant growth media are titrated to pH 5.6 using 1.0M KOH. Media are solidified with 1% w/v micro agar. For *Arabidopsis* cell culture media, pH value is titrated to 5.8 with 3% w/v sucrose, 0.5mg/L NAA and 0.05mg/L kinetin.

All chemicals (supplied by Fluka) were provided as follow:

MURASHIGE & SKOOG media (Murashige and Skoog, 1962):

A) 100x micro elements (1l) (need 10 ml of 100x in 1L)

<table>
<thead>
<tr>
<th></th>
<th>MW (g mol$^{-1}$)</th>
<th>Conc. (g l$^{-1}$)</th>
<th>Mol l$^{-1}$ in 1x</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_3$BO$_3$</td>
<td>61.83</td>
<td>0.62</td>
<td>1.002 x 10$^{-4}$</td>
</tr>
<tr>
<td>CoCl$_2$.6H$_2$O</td>
<td>237.93</td>
<td>0.00458</td>
<td>1.925 x 10$^{-7}$</td>
</tr>
<tr>
<td>CuSO$_4$.5H$_2$O</td>
<td>249.68</td>
<td>0.00233</td>
<td>9.339 x 10$^{-8}$</td>
</tr>
<tr>
<td>Na$_2$EDTA.2H$_2$O</td>
<td>372.24</td>
<td>4.125</td>
<td>1.108 x 10$^{-4}$</td>
</tr>
<tr>
<td>FeSO$_4$.7H$_2$O</td>
<td>278.02</td>
<td>2.78</td>
<td>9.999 x 10$^{-3}$</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>169.00</td>
<td>1.69</td>
<td>1.000 x 10$^{-4}$</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.2H$_2$O</td>
<td>241.95</td>
<td>0.025</td>
<td>1.033 x 10$^{-6}$</td>
</tr>
<tr>
<td>NaI</td>
<td>149.89</td>
<td>0.0749</td>
<td>4.999 x 10$^{-6}$</td>
</tr>
<tr>
<td>ZnSO$_4$.7H$_2$O</td>
<td>287.54</td>
<td>1.377</td>
<td>4.789 x 10$^{-5}$</td>
</tr>
</tbody>
</table>

B) 10x macro elements complete (1l) (need 50 ml of 10x in for 0.5x in 1L)

<table>
<thead>
<tr>
<th></th>
<th>MW (g mol$^{-1}$)</th>
<th>Conc. (g l$^{-1}$)</th>
<th>Mol l$^{-1}$ in 0.5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$ (anhydrous)</td>
<td>110.99</td>
<td>3.32</td>
<td>1.497 x 10$^{-3}$</td>
</tr>
<tr>
<td>MgSO$_4$(anhydrous)</td>
<td>120.37</td>
<td>1.80</td>
<td>7.506 x 10$^{-4}$</td>
</tr>
<tr>
<td>NH$_4$NO$_3$</td>
<td>80.04</td>
<td>16.50</td>
<td>0.0103</td>
</tr>
</tbody>
</table>
KNO₃ 101.11 19.00 9.396 x 10⁻³
KH₂PO₄ 136.09 1.70 6.245 x 10⁻⁴

C) 10x macro elements no P (1L) (need 50 ml of 10x in for 0.5x in 1L)

<table>
<thead>
<tr>
<th>MW (g mol⁻¹)</th>
<th>Conc. (g L⁻¹)</th>
<th>Mol L⁻¹ in 0.5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl₂ (anhydrous)</td>
<td>110.99</td>
<td>3.322</td>
</tr>
<tr>
<td>MgSO₄(anhydrous)</td>
<td>120.37</td>
<td>1.807</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>80.04</td>
<td>16.50</td>
</tr>
<tr>
<td>KNO₃</td>
<td>101.11</td>
<td>19.00</td>
</tr>
</tbody>
</table>

Sucrose: 3.0 g/L

50x MES, titrated with NaOH (2.35 mM in final 1x) to pH 5.6
(need 20 ml of 50x in 1L)

<table>
<thead>
<tr>
<th>MW (g mol⁻¹)</th>
<th>Conc. (g/L)</th>
<th>Mol L⁻¹ in 1x</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES</td>
<td>213.25</td>
<td>25.06</td>
</tr>
</tbody>
</table>

Johnson media (Johnson et al., 1957):

A) 10x macro elements complete (1L) (need 50 ml of 10x in for 0.5x in 1 L)

<table>
<thead>
<tr>
<th>MW (g mol⁻¹)</th>
<th>Conc. (g L⁻¹)</th>
<th>Mol L⁻¹ in 0.5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>101.11</td>
<td>6.067</td>
</tr>
<tr>
<td>Ca(NO₃)₂.4H₂O</td>
<td>236.15</td>
<td>9.446</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>115.03</td>
<td>2.301</td>
</tr>
<tr>
<td>MgSO₄(anhydrous)</td>
<td>120.37</td>
<td>1.204</td>
</tr>
</tbody>
</table>

B) 10x macro elements no P (1L) (need 50 ml of 10x in for 0.5x in 1 L)
<table>
<thead>
<tr>
<th></th>
<th>MW (g mol⁻¹)</th>
<th>Conc. (g L⁻¹)</th>
<th>Mol L⁻¹ in 0.5x</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>101.11</td>
<td>6.067</td>
<td>2.0 x 10⁻³</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>236.15</td>
<td>9.446</td>
<td>2.0 x 10⁻³</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>80.04</td>
<td>1.601</td>
<td>1.0 x 10⁻³</td>
</tr>
<tr>
<td>MgSO₄ (anhydrous)</td>
<td>120.37</td>
<td>1.204</td>
<td>5.0 x 10⁻⁴</td>
</tr>
<tr>
<td>KCl</td>
<td>74.56</td>
<td>1.491</td>
<td>1.0 x 10⁻³</td>
</tr>
</tbody>
</table>

The rest was the same with MS media.

2.2 Arabidopsis cell culture assays

2.2.1 Maintenance of Arabidopsis Cell Suspension Culture

Cells are grown at 27°C in continuous darkness in a 250 ml flask rotated at 130 rpm and are diluted by adding 2.0 ml to 50 ml of fresh MSS medium (Murashige and Skoog salt, 3% sucrose, 0.5 mg/L 1-naphthaleneacetic acid, 0.05 mg/L kinetin) every 7 days (Menges and Murray, 2002).

2.2.2 Arabidopsis cell starvation experiment

- On day one, pipette 2.0 ml Arabidopsis cell culture into flasks with 50 ml fresh MS liquid media. The number of the flasks is based on the experimental design;
- Put the flasks into 27 °C room under continues shaking & dark condition;
- On the fourth day, provide 2.5 mM KH₂PO₄ (final concentration) into each flasks;
- At day 7, take one sample for 0 time-point, mix all the cell culture together and split into 50 ml Falcon tubes. Each tube is contained around 30ml cell culture;
- Centrifuge the tubes at 800 rpm, 1 min with soft brake condition. Discard the supernatant and wash the cells with 0.2 mM CaCl₂/ 0.5 mM MES washing buffer (pH5.8) twice;
• Discard the supernatant again and wash the cells with fresh no Pi media;
• After centrifuge, discard the supernatant and add fresh no Pi media up to 50 ml per flasks;
• Pull all the cells into 1 L flask and mix them well;
• Split the cell culture into 250 ml flasks and start to take the time course.

2.2.3 Arabidopsis cell recovery experiment
• On day one, pipette 2.0 ml Arabidopsis cell culture into flasks with 50ml fresh MS liquid media. The number of the flasks is based on the experimental design;
• Put the flasks into 27 °C room under continues shaking & dark condition;
• On the fourth day, provide 2.5 mM KH₂PO₄ (final concentration) into each flasks;
• At day 7, mix all the cell culture together and split into 50ml Falcon tubes. Each tube is contained around 30 ml cell culture;
• Centrifuge the tubes at 800 rpm, 1min with soft brake condition. Discard the supernatant and wash the cells with 0.2 mM CaCl₂/ 0.5 mM MES washing buffer (pH5.8) twice;
• Discard the supernatant again and wash the cells with fresh no Pi media;
• After centrifuge, discard the supernatant and add fresh no Pi media to 50ml per flasks;
• Pull all the cells into 1 L flask and mix them well;
• Put the flask into 27 °C room under continues shaking & dark condition around two days;
• Split the cell culture into 250 ml flasks (50 ml per flask);
• Three hours later, provide chemicals in different flasks and start to take the time course.
2.2.4 Chemicals used for Arabidopsis cell culture experiments:

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Formula</th>
<th>Molecular Weight</th>
<th>Solubility</th>
<th>Concentration Stock</th>
<th>Concentration Working</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>KH₂PO₄</td>
<td>120.1</td>
<td>water</td>
<td>1.0M</td>
<td>2.5mM, 10.0mM</td>
<td>Fluka</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphite</td>
<td>KH₂PO₃ (2MH₃PO₃ :2MKOH=1:1)</td>
<td>136.1</td>
<td>water</td>
<td>1.0M</td>
<td>10.0mM, 20.0mM</td>
<td>Fluka</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>C₁₃H₂₃NO₄</td>
<td>281.3</td>
<td>ethanol</td>
<td>10.0mM</td>
<td>10.0uM,</td>
<td>Sigma</td>
</tr>
<tr>
<td>MG132</td>
<td>C₂₆H₄₁N₃O₅</td>
<td>475.6</td>
<td>DMSO</td>
<td>10.0mM</td>
<td>100uM</td>
<td>Piptide</td>
</tr>
<tr>
<td>E-64</td>
<td>C₁₅H₂₇N₅O₅</td>
<td>357.4</td>
<td>water</td>
<td>28.0mM</td>
<td>80uM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Cordycepin</td>
<td>C₁₀H₁₃N₅O₃</td>
<td>251.2</td>
<td>water</td>
<td>100mM</td>
<td>0.6M</td>
<td>Sigma</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>C₄₄H₆₈O₁₃</td>
<td>805.0</td>
<td>DMSO</td>
<td>300uM</td>
<td>300nM</td>
<td>Calbio</td>
</tr>
<tr>
<td>H89</td>
<td>C₂₀H₂₀BrN₃O₂S.₂HCl</td>
<td>519.3</td>
<td>DMSO</td>
<td>10mM</td>
<td>10uM</td>
<td>Sigma</td>
</tr>
<tr>
<td>K-252a</td>
<td>C₂₇H₂₁N₃O₅</td>
<td>467.5</td>
<td>DMSO</td>
<td>1mM</td>
<td>1uM</td>
<td>Calbio</td>
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<tr>
<td>Staurosporine</td>
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<td>DMSO</td>
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<tr>
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<td>100uM</td>
<td>Calbio</td>
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<td>DMSO</td>
<td>10mM</td>
<td>20uM</td>
<td>Sigma</td>
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</tbody>
</table>

2.3 Arabidopsis plant assays

2.3.1 Plant starvation experiment

- Arabidopsis seeds had been sterilized and put into cold room around 2 days;
- Pipette 5 seeds in each 250 ml flasks with 10ml 0.5x Johnson media;
- Set the flasks in plant growth room under long-day condition;
• Ten days later, pipette out the old media and change with the same fresh media;

• Three days later, pipette out the old media and use 10 ml 1.0 mM CaCl₂/5.0 mM MES solution to wash the flasks gently;

• Pipette out the wash buffer and add with 10 ml no Pi 0.5x Johnson or corresponding media into the corresponding flasks;

• Harvest roots and shoots samples separately under different time-course.

2.3.2 Plant recovery experiment

• Arabidopsis seeds had been sterilized and put into cold room around 2 days;

• Pipette 5 seeds in each 250 ml flasks with 10 ml 0.5x Johnson media;

• Set the flasks in plant growth room under long-day condition;

• Ten days later, pipette out the old media and use 10 ml 1.0 mM CaCl₂/5.0 mM MES solution to wash the flasks gently;

• Pipette out the wash buffer and add with 10 ml no Pi 0.5x Johnson media into the corresponding flasks;

• Three days later, pipette out the old media and change with the same fresh media;

• Three days later, change the media again;

• One day later, provide 2.5 mM KH₂PO₄ (final conc.) into the corresponding flasks;

• Harvest roots and shoots samples separately under different time-course.

2.3.3 Split roots experiment

• Arabidopsis seeds had been sterilized and put into cold room around 2 days;

• Grown the seeds on square plates with 0.5x MS media vertically under long-day condition in 23°C incubator;
Seven days later, cut the primary roots of seedlings just under the cotyledon and put the rest of the plants just under the cut line in the same plates around one week;

Till the new roots come out, select the plants which have two roots, wash the plant roots with no Pi media and transfer the seedlings to new -Pi vertical plates, till the roots grow around 10cm long;

Transfer the seedlings onto the three-compartment plates with -Pi half strength Johnson media (two components with liquid media, the other one with solid media, put the shoots on solid media, two roots into the different components with liquid media);

Let the plants growth on the three-compartments dishes for another week and Pi starved;

Add 2.5 mM KH$_2$PO$_4$ (final conc.) into one compartment with liquid -Pi media;

Harvest plant roots samples at different time-point (4 plates for each time-point).

2.3.4 $^{32}$P labelling plants

For Pi imagine:

Grow plants as usual in half strength MS media and then transfer to split roots Petri dishes with Pi-starved media. Let plants Pi starve for ~ one week on both compartments;

Pipette 6 µl $^{32}$P from the vial mixed with 10 ml de-ionized water in 15 ml falcon tube;

Add 500 µl labeled radioactivity Pi to the left part of dishes;

Harvest plant samples in each time point:

a) Remove liquid from left side of dishes into radioactive waste. Add 8 ml of 0.2 mM CaCl$_2$ (buffered to pH5.6 with MES) for one minute. Repeat it twice. Blot the plant briefly on absorbent tissue and place on a piece of filter paper;
b) Seal the samples with Saran membrane and air-dry the plants;

c) Use the Pi image cassette to count the radioactivity existing in the plants
(Short exposure is 3 hours, 3 days for long exposure).

For counting the radioactivity:

- Grow plants as usual in half strength MS media and transfer to split roots
  Petri dishes with Pi starved media. Let plants Pi starve for ~ one week on
  both compartments;

- Pipette 10 μl $^{32}$Pi from the vial and add to 8 ml de-ionized water in 15 ml
  falcon tube, Mix well (for 2 parallel experiment, 14 plants);

- Add 500 μl labeled radioactivity Pi to left side of dishes;

- Harvest samples from media:

- Pipette 10 μl aliquot of liquid media in both sides of dishes into scintillation
  vial;

- Harvest plant samples in each time point:

  a) Remove liquid from left side of dishes into liquid radioactive waste.
  Remove and discard into liquid radioactive waste. Add 8 ml of 0.2 mM CaCl$_2$
  (buffered to pH 5.6 with MES) for one minute. Replace with 8 ml half
  strength MS liquid media for one minute. Cut the two parts of roots and
  shoots with different blades separately, blot briefly on absorbent tissue and
  place into 1.5 ml tube.

  b) Weigh the roots and the shoots, put them into scintillation vial drying at 70
  °C about 2 days;

  c) After the samples are totally dried, pipette 1 mL 1% SDS into vials;

  d) Keep the vials at 60°C incubator more than 8 hrs;

  e) Add 10 ml scintillation solution into the vials, mix well and count with the
  scintillation counter.
2.3.5 Phosphate assay

The Pi content of the roots and shoots is determined using a phosphomolybdate colorimetric reaction according to the method of Chen (Chen et al., 1956) and adapted from Murphy and Piley method of phosphate determination (Murphy and Riley, 1962). Tissues previously frozen in liquid nitrogen have been put in safe-lock 2.0 ml tubes (from effendorf) with 2 metal balls and powered by shaker (from Retsch).

- Prepare reagent A: (store in cool, dark place)
  a) Dissolve 6 g ammonium molybdate in 125 ml de-ionized H2O;
  b) Dissolve 145.4 mg antimony potassium tartrate in 50 ml de-ionized H2O;
  c) Add both above solutions to 500 ml 5N H2SO4, mix and make up to 1 liter.
- Prepare reagent B: (make immediately prior to assay)
  Dissolve 105.6 mg L-ascorbic acid in 20 ml reagent A.
- Prepare a series of KH2PO4 standards (0, 2.5, 5, 10, 20, 30, 40, 50, 100 μM KH2PO4 in H2O);
- Add 190.5 μl reagent B to 1ml sample or Pi standard in 1.5 ml Effendorf tube;
- Ensure cap is screwed on tightly and place tubes in 55°C water bath for 1hr;
- Measure absorbance immediately at 880 nm.

Although this method measures Pi, it should be noted that there is some break-down of labile phosphate esters during this assay (Ames, 1966).

2.3.6 Root growth assay

Seedlings have been germinated and grown on square plates containing 75 ml half strength Johnson media (+Pi, -Pi, -Pi with 1.08% mannitol, -Pi with 20 mM KCl at 0.3% sucrose w/v). Seeds have been sown in a row 2 cm from the top of the plate. Plates have been sealed with parafilm and placed in a vertical position to encourage root growth along the surface of the medium. Growth conditions are a 16 h day / 8 h night photoperiod, at 21°C. The position of the primary root tip has been marked
after 7 days and then measured every subsequent 12 hours. Measurements from approximately 30 seedlings in each growth condition were pooled.

Similar growth conditions have been applied to determine root growth responses after transfer to starvation conditions. Seedlings have been germinated and grown on half strength MS media in vertical plates. On day 7, seedlings have been transferred to fresh vertical plates containing corresponding plates as shown previously. Roots have been rinsed in liquid media containing -Pi liquid media. Root tip position was marked on transfer and every 12 h after transferred.

2.3.7 Arabidopsis transformation by floral dipping

This procedure is based on a protocol described by Clough and Bent.

- Six pots of Arabidopsis seedlings (Wt. Col.) have been grown in short day condition (8 h light/16 h dark) for approximately 6 weeks, and then moved to long day condition (16 h light/8 h dark) to induce flowering;
- The first bolts to appear have been clipped off to encourage large numbers of secondary bolts;
- Once flowers formed, the plants were ready to transform;
- A 400 ml Agro-bacteria LB broth has been set up originally from transformed single clone by incubated overnight at 28°C with shaking at 225 rpm;
- The culture have been decanted into 250 ml centrifuge bottles and spun at 4000 rpm for 20 min at 4°C;
- The bacterial pellets have been resuspended into 1L of a 5% (w/v) sucrose solution containing 400 μl Triton-X 100;
- The plants have been dipped into the solution for 5 min whilst being gently rotated to ensure fully covered;
- Cover the pots with a plastic bag to increase humidity for two days;
- Harvest the seeds and select for transformants.
2.4 Genetic screen

2.4.1 Generation of reporter line

- After transform the corresponding plasmids (which contain \( \text{At4}::\text{LUC}, \text{Pht1}::\text{LUC}, \text{MGD3}::\text{LUC} \)) into plants, \( \text{T}_1 \) plants selected for \text{Hyg}';
- \( \text{T}_2 \) plants selected for 3:1 segregation;
- Choose 18 lines within 3:1 segregated lines;
- For each line, choose 9 \text{Hyg}' individuals and collected \( \text{T}_3 \) seeds;
- Selected for homozygous line;
- Choose the best one of which have the best kinetics of luciferase gene expression by providing Pi to Pi-deplete plants as the reporter line.

2.4.2 Generation the \( M_2 \) population

The reporter line seeds have been placed next to a \( \gamma \)-ray source for mutagenesis, which would give an absorbed dose of radiation of 300 Grays. Seeds have been sterilized and planted into soil. After eight weeks on soil, seeds from \( M_1 \) plants have been collected individually. This population can be used as \( M_2 \) seeds ready for genetic screen.

2.4.3 Genetic screen for \( \text{At4}::\text{LUC} \) reporter line

- Sterile \( \text{At4}::\text{LUC} \) report line homozygous (S49B) seeds and keep into cold room about 2 days;
- Put the seeds onto vertical plates with 1/2MS, 625 \( \mu \text{M} \) Pi, 0.6% sucrose, 1% agar, pH 5.6 in long-day condition incubator. Single layer nylon mesh (50 \( \mu \text{m} \)) is on the media and put the seeds on the mesh, two lines in each plate, 14-16 seeds per line;
- On the 4\(^{th} \) day, wash the nylon mesh & seedlings with no Pi media, then transfer the seedlings with nylon mesh onto no Pi vertical plates (1/2MS, 0.6% sucrose, 1% agar, pH 5.6);
- 8 days later, transfer the seedlings with nylon mesh onto the lid of the plates, saturate with 2 ml no Pi liquid media;
• Spray 1mM luciferin onto the plates and incubate the plates horizontally in long-day condition;

• On the next day (9th day), spray 0.2 mM luciferin first and take photos (Before take photos, the plates need to be kept in the dark ~5min).

• Then using 10mM Pi 40ml to wash the plate (2 min/each) and begin to count the time-course. Try to get rid of all the liquid on the plates, then spray 0.2 mM Luciferin and take photos at each time-point (2, 4, 8 hrs).

2.5 DNA analysis

2.5.1 PCR amplification of target sequences for cloning

Primers have been designed by Primer 3 programme (free on the internet http://frodow.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi ) to amplify target DNA sequences for cloning and to engineer enzyme sites for further cloning steps. Reactions have been performed in a T3 thermocycler (Biometra) using High Fidelity PLATINUM® Taq DNA polymerase (GibcoBRL). PCR products have been purified using Qiaquick spin kit (Qiagen).

2.5.2 Gene sequence

Long PCR products were cloned using the TOPO® cloning kit (Invitrogen). PCR products were incubated with the pCR®-XL-TOPO® plasmid vector, which was then transformed into One Shot® TOP 10 chemically competent cells. This was carried out according to manufacturers’ kits instructions.

2.5.3 DNA digestion with restriction endonucleases

DNA digestion with restriction endonucleases was carried out according to enzyme manufacturers’ recommended buffers and incubation temps.

2.5.4 Standard ligations

Ligations were performed using T4 DNA ligase (NEB) in the manufacturer’s buffer and incubated as recommended. Target insert to vector DNA ratios were approximately 3:1.
2.5.5 Transformation of Escherichia coli competent cells

Plasmid DNA was added to 100 μl chemically competent E.coli DH5α cells, at moment of thawing, and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 1 min., placed immediately on ice for 2 min, and then 1 ml of room temperature LB was added. Cells were incubated at 37°C, with shaking, for recovery. The cell suspension was centrifuged and the majority of the clear supernatant was removed before being resuspended in the residual supernatant and spread on fresh, selective LB plates.

2.5.6 Agarose gel electrophoresis

DNA separation on agarose gels was performed using gels made with and run in 0.5x TBE (45 mM Tris.HCl, 45 mM boric acid, 1 mM EDTA).

2.5.7 DNA isolation from agarose gels

DNA fragments from restriction digests were isolated from agarose gels using Qiaex II gel extraction kit (purchase from Qiagen).

2.5.8 Isolation of E. coli plasmid DNA

Bacterial colonies were screened for correct plasmid insertions by inoculating 3 ml LB (containing the appropriate selective antibiotic) with single bacterial colonies, which were grown overnight at 37°C with shaking. Plasmid DNA was isolated from overnight cultures by the alkaline lysis method. 1 ml culture was microfuged to collect bacterial pellet, the supernatant was poured off and the pellet was resuspended in the residual liquid. This was further resuspended in 250 μl buffer 1 (50 mM Tris, 100 mM EDTA, 400 μl RNaseA, pH 8). Cells were then lysed by adding 250 μl buffer 2 (200 mM NaOH, 1% SDS) and were left at room temperature for 5 min. The solution was neutralised by the addition of buffer 3 (2.55 M potassium acetate, pH 4.8) and mixed by gentle inversion. After centrifugation at 14000 rpm for 5 min, plasmid DNA was further purified by shaking with 200 μl phenol/ chloroform/ isoamylalcohol (25:24:1) mix and then centrifuging at 14000 rpm for 5 min. The aqueous phase was removed, precipitated with one volume of isopropanol and then centrifuged at 14000 rpm for 30 min. The supernatant was removed and the plasmid DNA pellet washed with 70% ethanol and centrifuged for 5
min. The supernatant was removed and the pellet air-dried then resuspended in 20 µl sterile H₂O. Once a bacterial clone with the correct plasmid insert was identified, purified plasmid DNA was isolated from a culture of the chosen clone using Qiaprep mini-prep kit (Qiagen).

2.5.9 Transformation of Agrobacterium tumefaciens

Plasmid DNA were added to 100 µl chemically competent A. tumefaciens GV3101 cells, at moment of thawing, and incubated on ice for 30 min. Cells were frozen in liquid nitrogen, then immediately heat-shocked at 37°C until thawed. 1 ml of room temperature YEP was added. Cells were then incubated at 28°C, with shaking, for 2 – 4 h. The cell suspension was centrifuged and the majority of the supernatant was removed, before being resuspended in the residual supernatant and spread on fresh, selective YEP plates containing appropriate selective antibiotics.

2.5.10 Isolation of total Agrobacterium DNA

Agrobacterium DNA was isolated to determine whether any rearrangements of the plasmid construct had occurred. 1.5 ml of the Agrobacterium culture used for the transformation of Arabidopsis, was centrifuged (14000 rpm, 1 min.). Pellets were resuspended in 300 µl TE. To lyse cells, 100 µl Sarkosyl in TE and 100 µl pronase in TE were added, and then incubated at 37°C for 2 h. The lysate was sheared by passing through a syringe. The aqueous phase was extracted twice with equal volumes of phenol saturated in TE. This was followed by extraction 2-3 times with equal volumes of chloroform. DNA was precipitated with 0.25 M NaCl and 2 volumes of ethanol at -20°C for 2 h. DNA was centrifuged for 5 min. at 14000 rpm. The supernatant was removed and the pellet was air-dried and then resuspended in 100 µl H₂O. Total Agrobacterium DNA was used to transform E. coli (only the plasmid DNA is incorporated). Plasmid DNA was isolated from E. coli and then digested with an enzyme that recognises multiple restriction sites in the plasmid. The resulting banding pattern was compared with that of the plasmid prior to Agrobacterium transformation.
2.6 RNA analysis

2.6.1 RNA isolation

The method of total RNA isolation was as follows for a 100 mg sample. The method was scaled up or down according to sample weight. Tissue was homogenised in TRIzol reagent (95 ml H₂O-saturated phenol (38%), 23.63 g guanidine thiocyanate (0.8 M), 7.61 g ammonium thiocyanate (0.4 M), 8.4 ml 3 M sodium acetate, pH 5 (0.1 M), 12.5 ml glycerol, plus H₂O to 250 ml). After addition of 200μl chloroform, samples were vortexed (15 s), left at room temperature for 2-3 min, then centrifuged at 14000 rpm for 15 min at 4°C. The aqueous phase was removed. To precipitate RNA from the aqueous phase, 0.8 M Na citrate/ 1.2 M NaCl (half the volume of the aqueous phase) was added, followed by the same volume of isopropanol, and then left at room temperature for 10 min. Samples were then centrifuged at 14000 rpm for 10 minutes, at 4°C. RNA pellets were washed with 1 ml 75% ethanol in DEPC-H₂O, briefly vortexed, then centrifuged at 14000 rpm for 10 minutes at 4°C. RNA pellets were briefly air-dried then resuspended in 50 μl DEPC-H₂O. RNA was re-precipitated with 2 M LiCl (final volume) overnight at 4°C, followed by centrifugation at 14000 rpm for 30 min at 4°C. RNA pellets were air-dried and then re-suspended in 50 μl DEPC-H₂O.

2.6.2 Quantitative RT-PCR analysis

RNA was reverse-transcribed using Invitrogen Life Technologies Superscript™ First-Strand Synthesis System for RT-PCR, using oligo (dT) primers. Approximately half a microgram of RNA was reverse-transcribed per half RT reaction. cDNA was diluted to 80 μl with sterile H₂O and 3 μl of diluted cDNA were used per 20 μl Quantitative PCR (QPCR) reaction. QPCR reaction components included 3 μl cDNA sample, 10 μl 2x Abgene QPCR Master Mix (containing 0.025 units/μl Thermo-Start® Enzyme), 4.2 μl sterile H₂O, 2 μl SYBR® Green 1 (1:10000 dilution of 10000x concentrate in DMSO, Molecular Probes), 0.4 μl (4pmol/μl) forward primer and 0.4 μl (4pmol/μl) reverse primer (see table 2.1). Each QPCR reaction was prepared in quadruplicate and then aliquoted into 96 well PCR plates, which were sealed with optical sealing tape. QPCR reactions were amplified using the iCycler iQ™ Real-Time Detection System (BIO-RAD), using the 490 nm filter for SYBR
green fluorescence detection. PCR reactions consisted of an initial 15 min. denaturation step (which was also necessary to activate the Thermo-Start® Enzyme) followed by 40 cycles of melting at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primer sequence for Quantitative PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>eIF 4A</strong> (cDNA)</td>
<td>Forward 5'TTCGCTCTTCTCTTTGCTCTC3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'GAACTCATCTTTGTCCTCAAGTA3'</td>
</tr>
<tr>
<td><strong>eIF 4A</strong> (genomic DNA)</td>
<td>Forward 5'CATTTTCTCCGCACATCATC3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'AACTGTGTGCGCTTCTGGTG3'</td>
</tr>
<tr>
<td><strong>At4</strong></td>
<td>Forward 5'GGATGGCCCAACACAAG3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'TAAACGGAAACAAATACACCAG3'</td>
</tr>
<tr>
<td><strong>PHO1.1</strong></td>
<td>Forward 5'CGATCAACTTACTAGCCAGATTCC3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'CACAAGTCTGTTTTTCTCTC3'</td>
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<tr>
<td><strong>MGD3</strong></td>
<td>Forward 5'TGCCACGTACATGTTC3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'TTGTCCTATGGATTCTTCTTTCAG3'</td>
</tr>
<tr>
<td><strong>PHO1.H1</strong></td>
<td>Forward 5'CACCTTTCTTACTTGCTTTTCA3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'CAGAGACTGGAGCAGCATC3'</td>
</tr>
<tr>
<td><strong>Pht1;1</strong></td>
<td>Forward 5'CTGCCAAGCTGATTAAGAGG3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'GACAGAGCACAAGAATCATTAC3'</td>
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<tr>
<td><strong>Pht1;4</strong></td>
<td>Forward 5'GGGACTCTCCCTCCCAAC3'</td>
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<td></td>
<td>Reverse 5'CATAGCAAAAACCGGAGAGA3'</td>
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<td><strong>IPT3</strong></td>
<td>Forward 5'CAAAACCACTTGCCTCTTT3'</td>
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<td></td>
<td>Reverse 5'GGACGAGATTCAATGGAGAGA3'</td>
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<td><strong>PEPC</strong></td>
<td>Forward 5'GAGTATTCCCCCTCGCTAC3'</td>
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<td></td>
<td>Reverse 5'CTTGGCGAACACCATTTC3'</td>
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<td><strong>IPS1</strong></td>
<td>Forward 5'TTGGGCAACTTATCCTTTTG3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'GCAAATTTACATGCACTGGTCTG3'</td>
</tr>
</tbody>
</table>

Table 2.1: Primer sequences for amplification of cDNA by Quantitative PCR

Each cDNA sample was amplified with eIF 4A (cDNA) primers to assess total RNA content. The eIF 4A gene encodes the constitutively expressed eukaryotic protein
synthesis initiation factor 4A (Metz et al., 1992). Amplification with eIF 4A (genomic DNA) primers was also performed to assess genomic DNA contamination when carrying out PCR with At4 primers (At4 mRNA is intron-less). All other primer combinations contained at least one primer designed to cross an intron border and thus only cDNA should have been amplified.

The threshold value was kept constant for all samples. To normalise for cDNA loading, the mean threshold cycle of amplification for each gene was subtracted from the mean threshold cycle of eIF 4A amplification from the same cDNA sample (this value was known as the ‘threshold cycle difference’). One sample per experiment was chosen as the base level to which all other samples were compared. This sample was always the sample with the largest calculated threshold cycle difference (i.e. the sample with the lowest gene expression). Therefore, the threshold cycle difference for each sample was subtracted from the largest threshold cycle difference value. The remaining value was the exponent of 2 (as each cycle involves the doubling of cDNA amounts of the previous cycle). This is simplified in the equation below:

\[
\text{Fold difference in gene expression} = 2^{c-(b-a)}
\]

\[
a = \text{mean threshold cycle of amplification for tested gene}
\]

\[
b = \text{mean threshold cycle of amplification for eIF 4A gene}
\]

\[
c = \text{largest threshold cycle difference value}
\]

2.6.3 Northern Blot Analysis

Separation of 10 μg RNA was performed in a 1% formaldehyde agarose gel as described in standard protocols. The gel and running buffer contained 1x MOPS. After electrophoresis, gels were washed with DEPC-H2O for 3 x 15 min, then 10x SSC (in DEPC-H2O) for 2 x 15 min, with slow shaking. RNA was blotted onto a nylon membrane overnight then UV cross-linked and immobilized at 90°C for 1 h. The nylon membrane was hybridized to a \(^{32}\text{P}\)-labelled 358 bp At4 cDNA fragment or 250bp Phl1;1 cDNA fragment separately. RNA loading was determined by hybridization to the constitutively expressed eukaryotic translation initiation factor, eIF4A. Blots were hybridized at 65°C and washed once at room temperature with 2x
SSC, 1% SDS, and once at 65°C with 2x SSC, 1% SDS, followed by 2 washes with 0.2x SSC, 1% SDS, at 65°C. Hybridization was quantified using a Phosphoimager.

cDNA was labelled by annealing At4 or Phl1;1 specific primers to denatured cDNA template and synthesizing new DNA strands using Klenow polymerase and (\(\alpha^{32}\)P) dCTP. A Sephadex-G50 column has been used to receive the purified DNA probe for hybridize with the nylon membrane. The hybridized probe on the membrane can be washed out by boiling in water for 30min.
3. Phosphate signalling in *Arabidopsis*

3.1 Summary

Plants can respond to different phosphate (Pi) availability in their environment. However, the molecular mechanisms involved in the early steps of Pi signalling are not thoroughly elucidated. In this chapter, experiments in *Arabidopsis* aimed to dissect both local and systemic responses to the perception of Pi are described. In Pi-deplete plants or cells, RNA abundance of Pi starvation induced (PSI) gene (the high-affinity phosphate transporter *Pht1;1*, the monogalactosyl-diacylglycerol synthase *MGD3* and *At4*, a phosphate starvation-induced gene of unknown function) are very rapidly regulated by provision of Pi. Phosphonate, a non-metabolic structural analogue of Pi, can activate the early response of the Pi signalling pathway, suggesting that phosphate perception, and not its metabolism, initiates Pi signalling. In the local Pi signalling pathway, *Pht1;1* RNA abundance appears exquisitely responsive to cytoplasmic Pi levels. Moreover, we propose the existence of a labile repressor protein or protein complex, which controls *Pht1;1* and *MGD3*, but not *At4* gene expression. This labile repressor(s) is regulated by ubiquitin-mediated proteolysis. We further demonstrated that protein kinases are involved in the early responses of Pi signalling. A conceptual framework of local Pi signalling is proposed in which the perception of Pi controls the stability of negative regulators of downstream responses.

To dissect the systemic signalling pathway, a split-root system was developed. By using the split-root system, we again illustrate that the Pi signal is initiated by Pi perception, but not metabolism, whilst the slow translocation of Pi in the split-roots plants demonstrates that Pi itself is not the systemic signal. By examining *MGD3* RNA abundance kinetics of Pi-deplete wild type and mutant plants in the split-root system, we find that *pho2* affects the early responses of Pi signalling pathway, while all other analyzed mutants are involved in its transport or are downstream effectors.

3.2 Introduction

Phosphate (Pi) is essential for metabolism and plays key regulatory and structural roles in plant cells, which have a high nutritional requirement for Pi (Marschner,
Phosphate is very tightly bound to soil minerals and difficult to assimilate. Hence most plants are chronically Pi starved (Raghothama, 2000). Adaptive responses to Pi starvation have been extensively studied, leading to a model in which the reduction of metabolic demand, mobilization of internal stores and the increase in Pi acquisition capacity are co-ordinately regulated to increase the likelihood for survival and successful reproduction (Raghothama, 2000; Abel et al., 2002; Hammond et al., 2003; Wu et al., 2003). However, these studies have not led to the identification of master regulatory genes coordinating adaptive responses to Pi starvation.

To dissect Pi signalling, very little is known at the molecular level in plants about how and where Pi cues are perceived, and which secondary signals and signalling pathways are involved in the regulation of adaptive responses. Mineral nutrients such as Pi have a dual nature – they are at once cues for signalling and materially required for metabolism. This has made it challenging to distinguish responses controlled by nutrient signalling pathways from those initiated through a material involvement of the mineral nutrient.

Previous studies have focused on plant gene expression responses following the withdrawal of Pi. However, the early responses at the molecular level are not easily distinguished. The timing and magnitude of adaptive responses to starvation are strongly dependent on the amount of Pi reserves in the plant, the rate of plant metabolism and the environment of the plant. The early signalling events after Pi withdrawal are masked by the plant’s efforts to maintain homeostasis, because of Pi accumulation and re-distribution. This approach does not directly target the initial events of Pi signalling.

In my study, a signalling pathway is defined as the initial perception of a cue and the subsequent processing of this information, but not the downstream effectors and their dependent genes, which constitute the effector pathway(s). Therefore, we analyzed the early responses of Pi signalling in the opposite scenario: When Pi was provided to Pi-deplete plants, the changes of Pi concentration in the environment would initiate Pi signals in the plants. The signalling processes would not be obscured, because rapid homeostatic adaptation occurs after the signal has been transported.
Analysis of the perception of Pi in Pi-deplete plants can directly target the immediate-early responses of Pi sensing and signalling mechanisms.

The reversible phosphorylation of proteins catalyzed by protein kinases and protein phosphatases is one of the major mechanisms by which living cells co-ordinate their responses to hormones, growth factors, cytokines and nutrients, as well as pathogens and other cell damaging agents. In the yeast $PHO$ system, protein kinases play central roles to control Pi signal transduction pathway (Ogawa et al., 2000). Unfortunately, searches of the $Arabidopsis$ genome have not revealed any closely related members of $PHO$ regulatory genes in yeast (Franco-Zorrilla et al., 2004). However, a number of small cell-permeant protein kinase and phosphatase inhibitors have been developed to study cell signalling for cancer research (Cohen, 1999). Working on the Pi signalling pathway with protein kinase or phosphatase inhibitors can directly guide us which types of biochemical mechanisms are involved in Pi signalling pathway.

Signalling pathways have two major functions. First, to amplify the input stimulus to yield an increased output (magnitude amplification) and second, to control how the magnitude of that amplification varies with the level of the stimulus (sensitivity amplification)(Koshland et al., 1982; Abel and Theologis, 1996). Such non-linear stimulus-response coupling is at the core of signalling. It is essential to filter noise and determine whether the outputs, and hence responses, are switch-like or more graded. In contrast, magnitude amplification only affects the absolute level of output, and thus of responses. It follows that mutational or pharmacological interference with core signalling functions will quantitatively affect stimulus-response coupling or alter the kinetics of responses. In contrast, impaired magnitude amplification is manifested in lower absolute, but similar relative levels of response. Using these criteria, we can conceptually distinguish between core signalling and effector (magnitude amplification) functions in Pi signalling by quantitative analysis of response kinetics. Similar approaches have previously been taken to dissect signalling pathways in animals (Herschman, 1991; Darnell et al., 1994).

Due to the tight coordination of Pi with soil minerals, Pi mobility is low and its distribution is highly heterogeneous even at very small spatial scales (Tinker and
Nye, 2000; Strawn et al., 2002). To efficiently assimilate and utilize available Pi, plant roots sense external Pi with high spatial resolution as well as the overall status of Pi nutrition within the plant. For example, individual trichoblasts respond to differences in external Pi by differential growth, regardless of the Pi-nutrition status of the plant, indicating that external Pi can be perceived cell-autonomously (Bates and Lynch, 1996). Plant cells can also sense internal, likely cytoplasmic, levels of Pi (Kock et al., 1998). Studies with split-root systems revealed that when Pi is available to only one half of a root system, starvation responses are not activated in the half lacking Pi. This implies the action of unidentified systemic signals to coordinate Pi acquisition and metabolism at the whole plant level to minimize costs associated with Pi assimilation (Liu et al., 1998b; Burleigh and Harrison, 1999; Baldwin et al., 2001).

Several Arabidopsis mutants have been identified that are affected in their responses to Pi starvation or in the ability to transport Pi. However, it is not known whether any of these mutations affect functions involved in the early step of Pi sensing or signalling. The pho1 mutant is impaired in xylem loading and hence the shoot is strongly Pi starved, while Pi uptake in the root is not strongly affected (Poirier et al., 1991). PHO1 encodes the first functionally characterized member of a novel class of membrane proteins, but it is not clear whether PHO1 regulates or mediates Pi transport (Hamburger et al., 2002). The pho2 mutant accumulates excess phosphate in shoots and is deficient in phloem transport from shoot to roots (Delhaize and Randall, 1995). It has been reported that PHO2 might encode an enzyme involved in proteolysis (Bari et al., 2003), but this has not been proven yet. The phrl mutant shows reduced responses to phosphate starvation and encodes a functionally conserved MYB-like transcription factor (Rubio et al., 2001; Wykoff and O'Shea, 2001). Recently, it was shown that the plant growth regulator cytokinin suppresses Pi starvation responses, mediated by the cytokinin receptor CRE1 (Martin et al., 2000; Franco-Zorrilla et al., 2002), and hence it has been proposed to function in Pi signalling.

Here we quantitatively examine the temporal patterns of gene expression in response to the perception of Pi in Arabidopsis. This analysis reveals two distinct effector pathways in the control of downstream responses to Pi perception at the cellular
level. One of these pathways controls immediate-early primary responses for which \textit{de novo} protein synthesis is not required. Expression of these primary response genes is partially under negative control. We show that the \textit{pho2} mutant affects Pi signalling, while all other mutants we characterized are impaired in transport or function as downstream effectors. Analysis of Pi translocation in Pi-deplete plants indicates that Pi itself does not appear to be the mobile systemic signal. These results validate the temporal and quantitative analysis of early signalling responses, in conjunction with the use of agonists and antagonists, as a powerful method to dissect plant nutrient signalling networks.
3.3 Results

3.3.1 Change in external Pi availability leads to rapid changes in gene expression

Previous studies have already shown that Pi-depleted *Arabidopsis* plants respond by repression of starvation-induced gene expression over several days following the provision of Pi and several downstream genes have been identified by using this approach (Bariola et al., 1994; Bates and Lynch, 2000a; Rubio et al., 2001).

To better characterize the early signalling systems involved in Pi sensing, we have determined response kinetics to Pi perception in Pi-depleted *Arabidopsis* roots and suspension cells, using a panel of molecular markers. Previous studies in our lab have already examined several Pi responsive genes expression under this condition (Whyte, 2004). Here, I focus on some of them. These markers include the high-affinity transporter *Pht1;1* (At5g43350) (Muchhal et al., 1996; Smith et al., 1997), the monogalactosyl-diacylglycerol (*MGDG*) synthase *MGD3* (At2g11810) (Awai et al., 2001) and the Pi-starvation induced *At4* gene (At5g03545) (Rubio et al., 2001). *Arabidopsis* plants and suspension cells were starved for 7 days or 2 days respectively (detail shown in Chapter 2), and then supplied with Pi (Fig. 3.1). As a control in real-time PCR, the ribosomal elongation initiation factor 4A (*eIF4A*) was amplified as representing a constitutive gene in all of my gene expression experiments (Metz et al., 1992).

**Fig 3.1.** Gene expression kinetics of phosphate abundance-responsive genes in *Arabidopsis* suspension-cultured cells and roots. Phosphate starved suspension cells (A) and whole plant roots (B) were exposed to 10mM Pi. Changes in transcript abundance of three representative genes *At4*, *Pht1;1*, and *MGD3* were monitored in a time course by real-time PCR. Error bars indicate standard error of the mean (SEM) and are smaller than the symbol when not visible.
As shown in Fig 3.1, the expression of these Pi-starvation induced gene was very rapidly down-regulated during the time course by provision of Pi. In Pi-depleted suspension cells, after addition of Pi, MGD3 gene expression consistently falls down to baseline levels within 2 hours, while At4 and Phl1;1 require exposure to Pi for 6 h to reach baseline levels (Fig. 3.1A). As Phl1;1 encodes the high-affinity transporter gene and is required to keep Pi homeostasis in cytoplasm (Mudge et al., 2002), expression of this gene does not respond too quickly to external Pi. At4 is expressed in stellar cells not directly exposed to the changes of external Pi concentration, in a pattern similar to AtIPS1 (Rubio et al., 2001), which suggesting its response to Pi in the environment might be much slower. Analysis of MGD3 mRNA abundance showed that gene expression is strongly induced by Pi deprivation and is generally higher in non-photosynthetic tissues with high levels of DGDG (digalactosyldiacylglycerol) in extra-plastidic membranes (Awai et al., 2001; Gaude et al., 2004; Kobayashi et al., 2004). It also has been suggested that MGD3 and other related genes are of conditional importance when the plants experience Pi stress: Activation of this pathway leads to the biosynthesis of a new DGDG pool, which is distributed from the plastid outer envelope to extraplastidic membranes (Benning and Ohat, 2005). The accelerated attenuation of MGD3 gene expression by provision of Pi to Pi-deplete plants likely due to the rapid translocation of Pi in cytoplasm.

In plants, the steady-state RNA levels of MGD3 reached basal levels between 10-30 min following their exposure to Pi (Fig. 3.1B), whilst the down regulation of Phl1;1 and At4 by provision of Pi required a much longer response time, normally several hours (data not shown). Either in Arabidopsis seedlings or suspension cells, MGD3 was consistently the gene most responsive to the perception of Pi, in terms of both velocity and magnitude. It has also been shown by micro-array analysis that genes coding for enzymes involved in galacto-lipid synthesis are highly activated at early stages of Pi deprivation in Arabidopsis (Julie et al., 2005). Therefore, in subsequent experiments, MGD3 gene expression was used as a reporter to analyze the early responses of Pi perception.

The fast down-regulation of marker gene expression suggests that the early responses of the Pi signalling pathway are under negative control by provision of Pi, and
perception of Pi does not require an extended effector pathway to restore MGD3 basal expression levels.

3.3.2 Pht1;1 RNA abundance directly responds to cytoplasmic phosphate level

To further analyze Pi perception and signalling, the kinetics of gene expression after withdrawal of Pi were also investigated previously in our lab (Whyte, 2004). As shown in Figure 3.2A, there was a transient increase of Pht1;1 gene expression 2-4 hours after transfer of Arabidopsis cells from +Pi media to -Pi media and which subsequently decreased. We did not observe this after transfer of cells from +Pi media to fresh media with different concentrations of Pi (Fig 3.2A) and such transient regulation did not appear with any other genes we tested (data not shown). In the quantitative analysis of RNA blot in similar experiments with Arabidopsis seedlings, the above observation was confirmed. At4 gene expression was used as a negative control (Fig. 3.2B).

Analysis of sub-cellular distribution of Pi pools with 31P-NMR has revealed that exposure of cells to Pi-deplete conditions initially results in a rapid drop in cytoplasmic Pi. Homeostasis by vacuolar reserves then occurs within a few hours, due to the kinetic properties of tonoplast transporters (Mimura, 1999). Therefore, our observation that Pht1;1 expression peaks transiently in plant within 2-4 hours after transfer to Pi-deplete media suggests that Pht1;1 RNA abundance responds directly to the changes of Pi concentration in cytoplasm, but not external Pi conditions. It indicates that this phenomenon is controlled by the early responses of local Pi signalling both in Arabidopsis cell culture and seedlings and could be used to as a marker for the changes of the cytoplasmic Pi concentration in the early response of Pi signalling. From Fig. 3.2, it also suggests that Pht1;1 might be have a role in cytoplasm to vacuole signalling to activate mobilization of vacuolar Pi stores.
Fig. 3.2 Pht1;1 RNA abundance directly responds to changes in cytoplasmic phosphate homeostasis. Arabidopsis cells or seedlings were in Pi media first, then washed with 1.0mM CaCl₂/MES and re-suspended in Pi-deplete media or corresponding media.

A: Pht1;1 gene expression of Arabidopsis cell culture; gene expression were monitored in a time course by real-time PCR. Error bars indicate standard error of the mean (SEM). B: Northern blot shown the Pht1;1 RNA abundance in Arabidopsis roots. Error bars show SEM.

3.3.3 Phosphonate (Phi) can mimic the early response of Pi signalling

Phi (HPO₃²⁻), also referred to as phosphorous acid or phosphonate, is an isostere of the Pi anion in which one of the oxygens bound to the P atom is replaced by hydrogen. Pi and Phi are acquired by plant cells via Pi transporters. Phi can suppresses the induction of Pi starvation responses in Pi-deplete Arabidopsis plants or Brassica rapa suspension cells when incubated over a period of days (Carswell et al., 1997; Ticconi et al., 2001; Varadarajan et al., 2002). Although the uptake and mobility of Phi and Pi are similar, there is no evidence suggesting that plants can utilize Phi as a sole source of Pi (Varadarajan et al., 2002). One of the distinct differences between Pi and Phi is that Pi can be assimilated into organic P compounds within minutes of uptake, whereas plants lack the ability to assimilate Phi (MacIntire et al., 1950; Guest and Grant, 1991). Furthermore, enzymes that catalyze the transfer of Pi groups can discriminate between Pi and Phi (Guest and Grant, 1991).

To examine whether Phi is involved in the early steps of Pi signalling, experiments similar to those in the first two sections were used in a short time course. As previous experiments showed, there is a transient peak of Pht1;1 gene expression 2-4 hours after transfer of the Arabidopsis cell culture to Pi-deplete media (Fig. 3.2).
After provision of different concentrations of Phi instead of Pi, this peak of *Phtl;1* gene expression does not occur within the 8 hours time course. The down-regulation of *Phtl;1* gene expression observed after provision of Phi, is slower than when providing Pi (Fig. 3.3A). In *Arabidopsis* Pi-deplete cells, *At4* and *MGD3* gene expression were also tested after providing 10mM Phi. Compared with providing 2.5mM Pi, these genes can be down regulated by provision of Phi, but the speed rate and magnitude is lower (Fig. 3.4).

To analyze the relationship between Pi and Phi molecules in the early responses of Pi signalling, different concentrations of Phi together with 2.5mM Pi were also used in Pi-deplete cells. The kinetics of *Phtl;1* gene expression showed that the concentration of Phi is inversely-proportional to the initial down-regulation velocity of gene expression (Fig. 3.3B). Our hypothesis is that Phi molecules can compete with Pi molecules to bind with Pi receptor(s) or transporter(s) and are recognized as Pi to inhibit of Pi starvation responses.

Based on our results, we conclude that the Phi molecule can be taken up as Pi in *Arabidopsis* cells, but cannot be used as Pi in Pi metabolism. This interpretation is also consistent with the results show that *PSI* gene expression starts to rise again at later time course in Pi-deplete cells after Phi treatment (Fig.3.4). The down regulation of Pi starvation gene expression caused by Phi is not as fast as when Pi is provided. The function of this molecule in the Pi signalling pathway is to generate a local signal and therefore, Phi can mimic the early response of Pi signalling and be used to analyze the early responses of Pi sensing.

![Figure 3.3](image-url)

**Fig. 3.3** Phosphonate (Phi) can mimic the early response of Pi signalling. *Arabidopsis* cells or seedlings were grown in low Pi media first, then washed with
1.0 mM CaCl₂/MES and re-suspended in Pi-deplete media or corresponding media. Pht1;1 gene expression was monitored in a time course by real-time PCR. Error bars indicate standard error of the mean (SEM) and are smaller than the symbol when not visible.

A: Pht1;1 gene expression in Pi-deplete media. B: Pht1;1 gene expression in Pi media.

Fig. 3.4 Gene expression kinetics of phosphate responsive genes in phosphonate or phosphate media.

A: Pht1;1, B: At4, C: MGD3. Phosphate starved Arabidopsis suspension cells were exposed to 2.5mM Pi or 10mM Phi. Gene expression was monitored in a time course by real-time PCR. Error bars indicate standard error of the mean (SEM) and are smaller than the symbol when not visible.

3.3.4 Negative regulation of phosphate response genes by labile repressors

In animal and plant systems, the immediate targets of signalling networks – primary response genes (Darnell et al., 1994; Abel and Theologis, 1996; Carswell et al., 1997) – are insensitive to the effects of protein synthesis inhibitors, such as cycloheximide (CHX). Recent work in plants have revealed a prevalence of negative regulation of plant hormone signalling networks by labile molecules susceptible to regulated protein degradation (Gazzarrini and McCourt, 2003). Meanwhile, in Pi-depleted conditions, the rapid down-regulation of our Pi responsive genes by
provision of Pi suggests that the early responses of the Pi signalling pathway might be under negative control. Therefore, we examined whether expression of _MGD3_, _At4_ and _Pht1;1_ is affected by CHX.

Treatment of _Arabidopsis_ suspension cells in the presence of adequate Pi with 20 μM CHX has no effect on _At4_ RNA level (Fig. 3.5A). _MGD3_ transcript levels are modestly elevated between one and four hours after addition of CHX, after which they drop again (Fig. 3.5A). In contrast, started from 2 hours after provision of CHX, _Pht1;1_ RNA levels are increased dramatically (Fig. 3.5A), suggesting that _Pht1;1_ RNA abundance is controlled by a labile repressor. After CHX blocks repressor protein synthesis, the concentration of repressor protein in cells becomes lower and _Pht1;1_ gene expression becomes higher. This hypothesis is also supported by the observation that after different concentration of CHX and 2.5mM Pi have been added simultaneously to Pi-deplete cells, _Pht1;1_ RNA levels are increased (Fig. 3.5B). The increased level of this response is dependent on the concentration of CHX (Fig. 3.5B) suggesting that a sizable initial pool of the labile repressor exists in the _Arabidopsis_ cell cytoplasm depending on CHX affection. After treating cells with CHX, no more new repressor protein can be made even under +Pi condition. As the repressor protein is presumably rapidly turned over, it can not accumulate and repress _Pht1;1_ gene expression.

Taken together, these results indicate that accumulation of _MGD3_ and _Pht1;1_ transcripts are regulated differently compared with that of _At4_ RNA. We concluded that steady state _Pht1;1_ RNA abundance is regulated by a labile repressor. _MGD3_ RNA abundance is also regulated by this repressor, but it is proposed that high magnitude sustained increases of _MGD3_ transcript abundance require an additional activator (Fig. 3.5A). The transient nature of _MGD3_ activation after CHX treatment suggests that this activator is either labile or limiting; hence _de novo_ synthesis is required. In contrast, _At4_ is not a primary Pi response gene.
Gene expression of phosphate-responsive genes after exposure of Arabidopsis cells to cycloheximide (CHX).

A: Pht1;1, At4 and MGD3 gene expression after providing 20 μM CHX; B: Pht1;1 gene expression after adding 2.5 mM Pi with different concentrations of CHX to Pi-depleted cells. Gene expression was determined by real-time RT-PCR. Error bars show SEM.

3.3.5 Ubiquitin-mediated proteolysis plays an important role in phosphate signalling

As described in the previous section, we obtained data that suggests the presence of a labile repressor in Arabidopsis cells that negatively regulates some early responses of Pi signalling. In eukaryotic cells, the ubiquitin-dependent proteasome pathway is one of the major mechanisms for the targeted degradation of proteins with short half-lives (Nawaz et al., 1999). We used different protein degradation inhibitors to test whether ubiquitin-mediated proteolysis is involved in the early steps of Pi signalling.

As shown in Figure 3.6A, Pht1;1 RNA abundance was down-regulated as expected after provision of 10 mM Pi to Pi-deplete cells. Interestingly, when cells were treated with 100 μM MG132 (a reversible, cell-permeable proteasome inhibitor), Pht1;1 RNA abundance was also down-regulated, but was not affected by treatment with E-64 (an irreversible cysteine protease inhibitor). As MG132 normally uses to reduce the degradation of ubiquitin-conjugated proteins by the 26S complex and E-64 works on cysteine protease covalently attached to hydrophilic acrylic beads via a 4-carbon spacer, it suggests that Pht1;1 RNA abundance might be controlled by the ubiquitin dependent signalling pathway.

Together with the CHX experiments, these results suggest that a labile repressor controls Pht1;1 gene expression. The abundance of this labile repressor is regulated by ubiquitin-mediated proteolysis to control Pht1;1 gene expression. MGD3 gene
expression was also tested in *Arabidopsis* cells: there is no obvious down-regulation of *MGD3* gene expression after treatment with *MG132* (Fig. 3.6B). This result suggests that *MGD3* transcript abundance is not directly regulated by ubiquitin-mediated proteolysis.

Even both of the difference between the MG132 and CHX experiments are really clear to show there is a feedback system to control phosphate transporter gene expression, it’s still worth well to use other ubiquitination inhibitors like MG115, Epoxomicin to confirm this pathway is really involved in the early responses of Pi signaling pathway.

![Fig. 3.6 Gene expression kinetics after exposure of Pi-deplete cells to protein proteolysis inhibitors.](image)

**A:** *Pht1;1* gene expression, **B:** *MGD3* gene expression. Gene expressions were determined by real-time RT-PCR. Error bars shown SEM.

3.3.6 The relationship between protein phosphorylation and phosphate signalling responses in *Arabidopsis*

The results from CHX experiments and the MG132 experiments suggest that the labile repressor(s) plays a significant role in the early responses to Pi sensing. It is proposed that this protein or protein complex receives a signal arising from Pi molecules in the environment and which controls immediate-early (*e.g.* Pi transporter) gene expression in the cytoplasm. Such a labile regulator(s) is likely to be an important primary target of the Pi signalling pathway. Therefore, identification and functional characterization this protein or protein complex are the next steps to focus on.
Protein phosphorylation and de-phosphorylation are one of the key mechanisms for the regulation of protein activity (Davies et al., 2000; Luan, 2003). Therefore, we decided to use phosphatase and kinase inhibitors to check whether protein phosphorylation or de-phosphorylation is involved in the early response of Pi signalling.

In these experiments, we provided Pi-deplete cells either with Pi alone, inhibitor alone or inhibitor together with Pi respectively to check whether the down-regulation of Pi responsive gene expression, caused by provision of Pi, was changed in Arabidopsis suspension cells. First, Pi-deplete cells were treated with okadaic acid (a protein phosphatase inhibitor) (Fig. 3.7 A). No obvious differences in Phd;1 gene expression kinetics between provision of Pi alone or provision of Pi and the inhibitor were observed (Fig. 3.7 A). This suggests that de-phosphorylation might not be a limiting step for the early steps of Pi signalling.

Pi-deplete cells were then treated with Staurosporine, a broad-spectrum kinase inhibitor, in the same way as in the previous experiment. Phl;1 gene expression did not have any changes after providing Staurosporine alone. Interestingly, after provision of Pi and Staurosporine together, Phl;1 gene expression kinetics were changed. The initial rate of Pi-induced down-regulated Phl;1 gene expression was attenuated when Staurosporine was supplied in addition to Pi (Fig. 3.7 B). This result shows that protein kinases may play a role in regulating Phl;1 gene expression in the early responses of Pi signalling.

Staurosporine is a potent, cell-permeable broad-spectrum protein kinase inhibitor. To examine which protein phosphorylation pathways might be involved in the early response of Pi signalling, different types of protein kinase inhibitors were used on Pi-deplete cells. Measurements were carried out for up to 8 hours after application of chemicals. This procedure let us focus on the early events of Pi signalling. Longer lasting effects are not taken into account, because they might results from effects on other metabolic pathways and possibly also involve complicated feedback effects. The experimental design was similar to earlier experiments with either Pi or inhibitors provided or together.
In Figure 3.7, kinase inhibitor H89 (protein kinase inhibitor) and Quercetin (Phosphatidylinositol (PtdIns) 3-kinase inhibitor) do not have obvious effects on Pht1;1 gene expression in Pi-deplete cells (Fig. 3.7 C, D). These data suggest that inhibitors H89 and Quercetin do not effect the labile repressor which is involved in the immediate-early responses of Pi signalling pathway to regulate Pht1;1 gene expression. As H89 inhibits protein kinase A pathway in mammalian cells and plants do not have PKA, it confirmed that H89 does not have effects on phosphate signalling pathway in plants.

However, after provision of the protein kinase inhibitors Roscovitine together with Pi, Pht1;1 gene expression was significantly different when compared to the provision of Pi alone (Fig. 3.7 E). Roscovitine is a member of the ATP-competitive cyclin-dependent kinase (CDK) inhibitor family. CDKs are a group of serine/threonine kinases that form active heterodimeric complexes by binding to their regulatory subunits, cyclins (Morgan, 1995). In the yeast PHO pathway, the Pi signal also regulates via a cyclin/cyclin-dependent kinase complex (CDK; Pho80/Pho85 protein complex) (Kaffman, 1994), as shown in Fig. 1.4. In plant cells, roscovitine acts also as a CDK inhibitor, characterizes restriction point and G2/M transition (Planchais et al., 1997).

Treatment of cells with Pi and Roscovitine together led to the complete suppression of the down-regulation of Pht1;1 gene expression normally observed after Pi treatments (Fig. 3.7 E). Pht1;1 gene expression does not respond to the addition of Roscovitine alone to Pi-deplete cells (Fig. 3.7 E). As expected, addition of Pi alone caused a decrease of Pht1;1 gene expression. This result indicates that provision of Roscovitine blocks the perception or sensing of Pi in an early step of the Pi signalling pathway. It is proposed that after Roscovitine and Pi are provided to Pi-deplete cells simultaneously, signalling initiated by the perception of Pi molecules is blocked at a Roscovitine-sensitive step and therefore, Pht1;1 gene expression is not down-regulated.

These results suggest that serine/threonine kinases are involved in the early stages of Pi signalling. I also tested MGD3 gene expression in these cells. Just as for Pht1;1, there was no response to provision of Roscovitine alone. After providing both Pi and
Roscovitine, the down regulation of \textit{MGD3} gene expression was slower than observed when Pi was provided alone (Fig. 3.7 \textit{F}). Together with previously observed differences when cells were treated with CHX, these results suggest that \textit{MGD3} transcript abundance is regulated different with \textit{Pht1;1}.

Similar experiments were performed with another kinase inhibitor – K252a. K252a is a member of a group of natural alkaloids, which act as kinase inhibitors by competing with the binding of ATP to the catalytic domain (Ruggeri et al., 1999). Interestingly, both of staurosporine and K252a have a stereoselective ring contraction of a pyranosylated indolocarbazole, it indicates there is a biosynthetic link between this two compounds (Stoltz and Wood, 1996). After treatment cells with K252a alone, \textit{Pht1;1} gene expression responded similar to the provision of Pi alone (Fig. 3.7 \textit{G}). Down-regulation of \textit{Pht1;1} expression by Pi was unaffected by the presence of K252a. These results suggest that K252a affects the early steps of Pi signalling. However, we do not know exactly how this inhibitor interferes with the Pi signalling pathway and which protein kinase K252a inhibits. One hypothesis is that K252a can stabilize the labile repressor by inhibiting a protein kinase-dependent step involved negative control repressor protein or protein complex. After addition of K252a, the labile repressor would not be degraded and therefore accumulates in cells leading to a repression of \textit{Pht1;1} gene expression. However, we cannot rule out other possibilities. For instance: because the labile repressor(s) itself is regulated by ubiquitin-mediated protein degradation, K252a might inhibit proteolysis. In this case, the repressor(s) would not be degraded after provision of K252a and therefore suppresses \textit{Pht1;1} gene expression. It is also possible that the non-phosphorylated form is the active form of repressor protein required to accomplish its function. If K252a inhibits repressor protein phosphorylation, the functional repressor will accumulate in cells and negatively control \textit{Pht1;1} gene expression.

Therefore, we conclude that protein kinases are involved in the immediate-early steps of Pi signalling pathway. Protein kinase inhibitor Roscovitine and K252a inhibit different phosphorylation steps in regulating Pi transporter gene expression. Even from the inhibitor experiments, we could not find any targets of the inhibitors in the Pi signalling network. Still by using more than one inhibitors can potentially
help us to describe the frame of the Pi signalling pathway (MacKintosh and MacKintosh, 1994).

Fig. 3.7 MGD3 and Pht1;1 gene expression after exposure of Pi-depleted cells to kinase inhibitors. Genes expression were determined by real-time RT-PCR. Error bars shown SEM. A: Okadaic acid, B: Staurosporine, C: H89, D: Quercetin, E: Roscovitine, F: Roscovitin (MGD3), G: K252a.
3.3.7 Systemic phosphate signalling.

As well as the responses of the local Pi signalling pathway, we are also interested in
the systemic responses to Pi perception in Arabidopsis seedlings. Therefore, we
initiated a novel Arabidopsis split root system to distinguish local signalling from
systemic signalling.

In this system, a three-compartment Petri dish was used and one seedling was grown
on each plate. One of the compartments contains solid media with no added
phosphate. The other two contain liquid media in which the composition can be
changed individually (Fig. 3.8). Root systems of seedling were split into two parts
so that each half of the root system is placed in separate compartments with liquid
media, while the shoots part of the seedlings sit on top of the solid media (Fig. 3.8).
Following an initial period of growth, Pi was depleted from the entire root system to
induce Pi starvation. At t=0, one compartment only was provided with 2.5mM Pi.
During the time course, the roots in both compartments were harvested and real-time
RT-PCR was used to analyze the molecular changes caused by the Pi signal
travelling from one part of the roots (Pi-replete) across the shoots to the Pi-deplete
half of the root system. The roots in the Pi-replete compartment demonstrate local
responses, while the roots in the Pi-deplete compartment show systemic responses.
Therefore, by using this system, we can analyze local Pi signalling and systemic Pi
signalling simultaneously.

The attenuation of MGD3 RNA abundance in the Pi-replete part of the root system
shows response kinetics similar with those have been observed in whole plant assays
(Fig. 3.1; Fig. 3.8). Remarkably, steady-state MGD3 RNA levels in the part of the
root system not exposed to Pi fell at an only slightly lower rate compared to the Pi-
replete root (Fig. 3.8). These results indicate that a signal required for systemic
down-regulation of MGD3 transcripts must move very rapidly through the severely
Pi-deplete plant.

Then we used 10mM Phi instead 2.5mM Pi in this experimental system (Fig.3.8).
MGD3 RNA level in the Phi-treated part of the root system showed response kinetics
similar to those observed when exposed to Pi molecules (Fig. 3.8). MGD3 transcript
levels in the part of the root system not exposed to Phi dropped at a low rate,
reaching ~90% attenuation after 6 hours. The attenuation rate of this response indicates that the systemic signal initiated by Phi, just like Pi, is rapidly transmitted through the plant and this systemic signal is initiated by perception of Pi, but not by Pi metabolism.

![Graph showing MGD3 expression](image)

**Fig. 3.8** Perception of phosphate, not phosphate metabolism, initiates phosphate signalling.

Phosphate-starved plants with split root systems were exposed to 2.5mM Pi or 10mM Phi on left side and *MGD3* gene expression was determined in a time course (Left panel). The panel on the right shows the split root growth system.

There are three separate compartments in this Petri dish. One of the compartments contains solid media with no added Pi. The other two contain liquid media the composition of which can be changed individually. Root systems of the seedling have been split into two parts so that each half of the root system is placed in separate compartments with liquid media, while the shoot of the seedlings sit on top of the solid media.

### 3.3.8 Slow translocation of phosphate to systemic roots

Through the analysis of the split root system, we showed that the systemic signal moves quite rapidly through the plant and that systemic signalling is initiated by Pi perception. However, we do not know what the Pi signal is. As Pi and Phi are transported across the plasma membrane by Pi transporters, and are mobile throughout the plant (Carswell et al., 1997), it cannot be excluded that phosphate or phosphonate are systemic signals.

To address this question in more detail, Pi-deplete plants in the split root system were exposed to $^{32}$P-labeled orthophosphate. We then followed the movement of radioactivity throughout the plant. Using this approach, we examined how Pi moves from the Pi-replete to the Pi-deplete half of the root system. If Pi is the systemic
signal, the radioactivity should move from the Pi-replete part into the roots part that does not contain any $^{32}\text{Pi}$. 

Significantly, up to 4 hours after providing $^{32}\text{Pi}$ in left part of roots, there was no detectable $^{32}\text{Pi}$ accumulation in the Pi-deplete part of the root system on the right (Fig. 3.9). However, based on the molecular analysis in the last section, we know that the systemic signal should be there (Fig. 3.8). Based on the slow translocation of $^{32}\text{Pi}$ to the systemic roots, we conclude that Pi itself is likely not the systemic signal. Interestingly, from the localization of $^{32}\text{Pi}$ in plant, we observed that in the shoot, stems, pedicels and floral meristems were a stronger sink than leaves, which only accumulated $^{32}\text{P}$ from 240 minutes onwards (Fig. 3.9).

**Fig. 3.9** Slow systemic movement of phosphate within split root system.

*Arabidopsis* plants were grown with split root systems. Pi-deplete plants were provided with $^{32}\text{P}$ labelled orthophosphate to the left half of the root system and samples removed at different time courses. The systemic half of the root system not exposed to $^{32}\text{P}$ is not visible in this Phosphor image exposure.

3.3.9 Responses to phosphate perception are altered in mutant backgrounds

We hypothesized that impaired perception or signalling of a Pi stimulus would primarily affect stimulus-response coupling, which would reveal itself in an initial velocity or relative magnitude of the response distinct from that observed in wild type plant. In contrast, a genetic defect in any effector pathway initiated by the perception of Pi, is proposed to primarily affect the absolute magnitude of gene expression. To examine whether any of the mutants, previously reported to be affected in Pi starvation responses (Burleigh and Harrison, 1999; Baldwin et al., 2001; Rubio et al., 2001; Hamburger et al., 2002; Varadarajan et al., 2002) are
impaired in Pi signalling, we analyzed their response kinetics in the split root systems, to simultaneously monitor local and systemic responses.

Analysis of the local responses revealed that all genetic backgrounds we examined responded with very similar MGD3 expression kinetics when compared with the wild type. The only marked exception was pho2 mutant (Fig. 3.10A). Attenuation of MGD3 transcript abundance in the pho2 background consistently proceeded more slowly in response to the perception of Pi and never reached the baseline levels observed in the other backgrounds, even after prolonged exposure to Pi (data not shown). This result suggests that the PHO2 gene is involved in local Pi signalling.

Analysis of the systemic responses revealed further differences between the various genetic backgrounds (Fig. 3.10B). Wild type, phr1 and crel-2 showed very similar MGD3 RNA response kinetics, with crel-2 responding slightly more slowly and to a lesser magnitude than the former two. These observations suggested that PHR1 and CRE1 are not involved in Pi signalling per se, but rather function further downstream. This was confirmed when we compared absolute levels of local and systemic changes in MGD3 RNA abundance between these mutants (data not shown): Pi starvation-induced levels of MGD3 RNA in phr1 and crel-2 plants were only 16 and 42%, respectively, of the wild type response. Moreover, following exposure to Pi, MGD3 transcript levels remained lower than corresponding wild type levels.

As expected, attenuation of MGD3 RNA abundance in systemic roots in response to Pi exposure of the other half of the root system was much reduced in the phol background (Fig. 3.10B). Although phol is impaired in xylem loading of Pi, when exposed to high external Pi concentrations, Pi can be translocated to the shoot and rescue the mutant phenotype (Burleigh and Harrison, 1999). MGD3 RNA levels in Pi-deplete phol plants were 72% higher than in wild type, consistent with increased starvation caused by reduced Pi transport in this background. MGD3 expression in Pi-deplete wild type or pho2 plants was very similar, but the kinetics of attenuation of MGD3 RNA levels in response to Pi exposure were markedly reduced in the pho2 mutant, in terms of both rate and magnitude (Fig. 3.10B). These data suggest that
PHO2 is involved in Pi stimulus-response coupling whereas e.g. PHR1 is involved in magnitude amplification.

Fig. 3.10 Gene expression kinetics of MGD3 in wild type and Arabidopsis mutant plants grown with split root systems. One half of the root system of starved plants was exposed to phosphate and MGD3 RNA abundance was measured in exposed roots (A), and in non-exposed, systemic roots (B). MGD3 RNA levels were plotted relative to the initial, fully starved levels at t=0 for each individual genotype (A). Error bars shown SEM and are smaller than the symbol when not visible.
3.4 Discussion

Previous studies have focused on plant responses to the withdrawal of Pi i.e. the onset of Pi-starvation responses. However, the timing and magnitude of adaptive responses to Pi starvation are strongly dependent on previous exposure to Pi, the rate of plant metabolism and the environment of the plant. More importantly, this approach does not directly target the initial signalling events. We defined a signalling pathway to include the initial perception of a cue and the subsequent processing of this information, but not the downstream effectors and their dependent genes, which constituted the effector pathway(s). Therefore, we analysed immediate-early responses to the perception of Pi in Pi-deplete plants or suspension cells, which have revealed novel features of Pi signalling.

3.4.1 Mode of regulation

Our data shows by temporal and quantitative analysis in Pi-deplete Arabidopsis plants and cells that perception of Pi leads to very rapid suppression of starvation-induced gene expression (Fig. 3.1). The rapid down-regulation of the expression of marker genes suggest that the early steps of the Pi signalling pathway are under negative control, and that responses to the perception of Pi does not require an extended effector pathway. In terms of both velocity and magnitude, MGD3 is consistently the gene most responsive to the perception of Pi in the environment. Micro-array data has also shown that genes coding for enzymes involved in galactolipid synthesis are highly activated at early stages of Pi deprivation in Arabidopsis (Julie et al., 2005). Analysis of MGD3 RNA abundance has shown that gene expression is of conditional importance when the plants experience Pi stress leading to accelerate the biosynthesis of di-galactosyldiacylglycerol (DGDG) (Benning and Ohat, 2005).

In order to distinguish Pi perception from its metabolism, we used Phosphonate (Phi), a non-metabolic structural analogue of Pi as a cue to initiate Pi perception. We show that Phi generates a local signal (Fig. 3.3 A), that does not involve Pi metabolism (Fig. 3.3B & Fig. 3.4). Based on its distinct response kinetics to the Pi analogue phosphonate (Phi), Phi;1 RNA abundance was found to very sensitively respond to cytoplasmic Pi levels, not to extracellular levels (Fig. 3.2). Pi homeostasis
in the cytoplasm is one of the most important aspects of Pi metabolism. Meanwhile, these data also revealed that perception of Pi, not its incorporation into metabolites, is the crucial event in Pi signalling. Hence, it is not obvious that plasma membrane-intrinsic Pi transporters are involved in local Pi signalling, as has been proposed in yeast (Giots et al., 2003).

3.4.2 Mechanisms of local signalling

The observed rate and magnitude of attenuation of *Pht1;1* and *MGD3* transcript levels after perception of Pi by Pi-deplete cells suggests that this response is under negative control (Fig. 3.1). This view is also supported by the CHX experiments that Pi signalling pathways act by negative control to regulate the RNA abundance of Pi starvation response genes (Fig. 3.5). A similar conclusion was reached based on studies of proteins that bind to promoter sequences of Pi starvation-induced genes (Ogawa et al., 2000). Enhanced accumulation of *Pht1;1* and *MGD3* RNA in response to CHX treatments (Fig. 3.5A), and the increase of *Pht1;1* RNA in the presence of both Pi and CHX (Fig. 3.5B), indicate that *Pht1;1* RNA abundance is under control of a labile repressor. Negative control by labile repressors is widespread in the control of plant hormone signalling pathways (Inoue et al., 2001).

Furthermore, the attenuation of *Pht1;1* RNA levels when cells are treated with a proteasome inhibitor suggests that the degradation of the labile repressor is controlled by the ubiquitination pathway (Fig. 3.6). Ubiquitin-mediated proteolysis has been confirmed to be a crucial regulated pathway for plant development and their responses to the environment. It must be very specific and tightly controlled, any degradation of the wrong protein or an error in the timing of this process could prove catastrophic for an organism (Sullivan et al., 2003).

Phosphorylation and dephosphorylation of a protein often serve as an "on-and-off" switch in the regulation of its activities. Recent studies demonstrate the involvement of protein phosphorylation in almost all signalling pathways in plants (Luan, 2003). For example, F-box proteins for ubiquitin-dependent proteolysis have been shown to capture phosphorlyated substrates by means of carboxy-terminal protein–protein interaction regions, such as WD40 repeat domains or leucine-rich repeat (LRR) domains (Skowyra et al., 1997; Nash et al., 2001).
Treatment of Arabidopsis suspension cells with kinase or phosphatase inhibitors can help us to characterize the early responses of the Pi signalling pathway. By treatment of cells with Pi together with different phosphatase inhibitors or kinase inhibitors in Pi-deplete cells, the kinetics of Phl1;1 gene expression have showed different effects from protein kinase inhibitors Roscovitine or K252a (Fig. 3.8 E,G).

3.4.3 Model of local Pi signalling

Based on the previous results, we have developed a model for immediate-early Pi signalling (Fig. 3.11). In this model, Phl1;1 gene expression is under negative control by protein kinases and one or several repressor proteins, as the CHX and kinase inhibitor experiments show (Fig. 3.5 & 3.7). MGD3 RNA abundance is regulated not only by such labile repressor(s) but also a proposed activator (Fig. 3.5A & 3.7F). Furthermore, the degradation of this labile repressor protein or protein complex is likely mediated by the ubiquitin-dependent proteolysis, as the MG132 experiment shows (Fig. 3.6).

In our model (Figure 3.11), when Pi or Phi is presented to the cells, the Pi signal can repress the activity of one or more K252a-sensitive protein kinases, which results in a stabilization the repressor protein. Therefore, Phl1;1 gene expression is down regulated by the accumulation of the repressor protein or protein complex in the cells. At the same time, the MGD3 gene expression is regulated by an activator and indirectly controlled by the labile repressor. Under Pi-deplete condition, the labile repressor does not accumulate, as no Pi exists to inhibit the protein kinases which are proposed to phosphorylated the repressor protein(s), which leads to their destabilization; therefore, Phl1;1 RNA is expressed at a high level. Because of low accumulation of the repressor protein in the cells, MGD3 gene expression is highly induced by the activator.

The CDK inhibitor Roscovitine is a potent and selective inhibitor of ATP-competitive cyclin-dependent kinases (Planchais et al., 1997). CDKs play key roles in the progression of the cell cycle in eukaryotes and have important significances underlying several essential processes, including cell fate determination, differentiation, organ development, growth, cell death, carcinogenesis, etc (Umeda et al., 2005). As shown in Fig. 3.11, Roscovitine can inhibit the kinase(s) to
phosphorylate their substrates and stabilize its function to negative control repressor protein. Even if Pi is presented, the Pi signal cannot be perceived and the repressor protein is degraded by the ubiquitin-mediate proteolysis and $Phl1;1$ RNA abundance remains high (Fig. 3.7 E, provision both Pi and Roscovitine). This data also clearly suggests that K252a and Roscovitine interfere with the early-response of Pi signalling pathway in different ways. But there still remains an unresolved issue: question of $MGD3$ gene expression in provision of Pi and Roscovitine to the cells: even if Roscovitine blocks the Pi signal through our model, the down regulation of $MGD3$ RNA abundance is occurred (Fig. 3.7 F, provision both Pi and roscovitine). I do not have an answer yet. It might be because of the $MGD3$ RNA is not stable or because plant has developed some other ways to transfer the Pi signal, compared with the $CDK$-dependent $PHO$ system in budding yeast. So Roscovitine alone is not sufficient enough to totally block the Pi signal.

In the model, K252a may act through this pathway to stabilize the repressor protein or protein complex. Provision of K252a to the cells can attenuate $Phl1;1$ RNA abundance (Fig. 3.7 G). Still there also has a problem with the $MGD3$ RNA abundance is not quite down regulated by the accumulation of the repressor protein due to the provision of K252a (data not shown). Even we know that K252a interacts at the ATP-binding site of protein kinases, a detailed characterization of the specific phosphorylation step still needs further analysis.

Our model of Pi signalling has been tested using protein kinase inhibitors. These data suggest that kinase(s) are involved in the early responses of Pi signalling pathway as our model shows (Fig. 3.11). However, there also exist some problems of which are quite difficult to explain in our model. These questions might be due to the problems from our model itself, but they indicate the limitation of the experimental approach - The inhibitor approaches are not so easy to ascribe to a specific signalling pathway. We have to identify more elements in this signalling pathway, some approaches just like the genetic screen in Chapter 5.
**Fig. 3.11** Model for local phosphate signalling.

Pi molecule is acted through kinase(s) and repressor to down-regulate *Pht1;1* gene expression (three negative arrow). This repressor protein is degraded by the ubiquitin-mediated proteolysis (negative arrow). *MGD3* RNA abundance is indirectly controlled by the repressor through an activator protein. Inhibitors are involved in this model through different positions. Blunt arrow shows negative regulation. Thin arrow shows positive regulation.

### 3.4.4 Systemic phosphate signalling

Systemic Pi signalling was also characterized in my results by using the split root system. We illustrated that the Pi systemic signal is initiated by Pi perception, but not metabolism. Meanwhile, the slow translocation of Pi in the split-root plants demonstrates that Pi itself is not the systemic signal. By examining *MGD3* RNA abundance kinetics of Pi-deplete wild type and mutants in split-root system (**Fig. 3.10**), we found that the *pho2* mutant affects the early responses of the Pi signalling pathway, while all the other analyzed mutants were involved in its transport or are downstream effectors.

*PHO2* possibly encodes an ubiquitin conjugating enzyme (E2) involved in regulated proteolysis (Bari et al., 2003). Our CHX results suggest that *de novo* protein synthesis is required to negatively regulate *Pht1;1* abundance in the presence of Pi (**Fig. 3.5A, B**). We propose that *PHO2* is involved in the signalling pathway by negatively regulating the abundance of effector proteins by promoting their
degradation in the absence of Pi (Fig. 3.11). Pi signalling is not completely repressed in the pho2 mutant, this may be because pho2 is not a null allele, or because of genetic redundancy, as there are 45 E2 and E2-like genes in the Arabidopsis genome (Vierstra, 2003). If proteolysis is required to attenuate Pi-starvation responses in the presence of Pi, then impaired proteolysis would explain enhanced accumulation of Pi in pho2 shoots, as the expression of Phil;1 would not be appropriately repressed, even in the presence of adequate Pi.

The rate of transport and localization of Pi in Pi-deplete plants with split root systems, suggests that Pi translocation generates the systemic signal to coordinate the Pi budget of whole plants. A systemic signal has previously been postulated to explain why Pi-starvation responses in non-exposed roots were suppressed when only part of the root system was exposed to Pi. Although numerous studies had shown Pi to readily move in the plant (Rausch and Bucher, 2002), it had been proposed not to be the systemic signal (Burleigh and Harrison, 1999).

Here, we show that 32Pi supplied to one half of a previously starved split-root system does not accumulate in the non-exposed half even 4 hours later (Fig. 3.9). The largely unimpaired systemic response observed in crel-2 and crel-4 (data not shown) mutants also suggests that cytokinin is not an essential systemic signal, although we cannot exclude redundancy with other histidine kinases (Kakimoto, 1996). As we cannot rule out incorporation of the radiolabel into another molecule, we conclude that any systemic signals are not likely to incorporate Pi. Furthermore, when we only partially starved the root system, then withdrew Pi completely from one side and provided the other side with adequate Pi, it took several days to attenuate starvation-induced gene expression (Whyte, 2004). This argues that systemic responses to Pi are not controlled by a distinct signal for phosphate but rather by the sink strength for phosphate in the distant organ. Because shoot to root transport of Pi is suppressed in Arabidopsis under conditions of Pi starvation, while root to shoot transport is enhanced (Dong et al., 1998), this suggests that it is the magnitude of Pi demand in the non-exposed root that determines the direction and destination of Pi flux (Fig. 3.8). Thus, systemic Pi signalling is governed by the signal from the source, not by demand in the distant organ.
3.4.5 Comparison with yeast

Pi signalling, in terms of both regulatory molecules and signalling logic is currently best understood in *S. cerevisiae* (Ogawa et al., 2000; Giots et al., 2003). Under high Pi concentration, the cyclin-dependent kinase-cyclin pair *PHO80/PHO85* is active. The master effector gene, the transcription factor *PHO4* is fully phosphorylated and localized to the cytoplasm, and transcription of Pi-responsive genes such as acid phosphatase *PHO5* and Pi transporter *PHO84* is turned off. In response to Pi limitation, *PHO80/PHO85* is inactivated, which in turn is held in check by the cyclin-dependent kinase inhibitor *PHO81*, and unphosphorylated *PHO4* accumulates in the nucleus where it activates transcription of Pi-responsive genes such as *PHO84* and *PHO5*. The activity of *PHO81* is controlled by upstream sensing components, which include phosphate transporters and protein kinase A. The central mechanism of negative regulation in yeast is protein phosphorylation to control the sub-cellular localization of *PHO4* (Ogawa et al., 2000; Giots et al., 2003; Thomas and O'Shea, 2005), model shown in Fig. 1.4.

In *Arabidopsis*, we propose this mechanism to be Pi sensing-dependent regulation of protein stability. Just as our model shows, the labile repressor protein stability is negative controlled by a Pi induced sensing system (*Fig. 3.11*). Control of subcellular localisation may also play parts in plants. It remains to be determined whether Pi signalling directly controls proteasome activity. Alternatively, Pi signalling acts through protein phosphorylation of cognate substrates to control substrate recognition by F-box proteins involved in proteolysis (Vierstra, 2003). The phenomena in yeast indicate that a similar signalling pathway might also exist in *Arabidopsis* and provide hints to find the key components in Pi signalling pathway.
4 Carbon and nitrogen availability dominate phosphate starvation responses via control of plant growth in Arabidopsis

4.1 Summary
Responding appropriately to nutrient availability is of fundamental importance for plant growth and adaptation to the environment. We examined whether a plant responds to phosphate based on its demand for phosphate, plants were grown at varying concentrations of carbon (C) and nitrogen (N), which promote root or shoot growth, respectively. We also used cytokinin treatments, which like N, inhibit root growth but promote shoot growth. We found that increased carbon enhances phosphate (Pi) starvation responses in whole plant, while nitrogen and kinetin treatments suppressed these responses in roots. The effects of carbon on phosphate starvation-induced gene expression are similar in roots and shoots, while nitrogen or cytokinin (CK) availability has opposite effects in shoots and roots. Elevated nitrogen or cytokinin availability, shifts shoot-root mass ratios in favour of shoots and alter cellular Pi concentration in both tissues, while increased carbon specifically promotes root growth. Our data suggest that the carbon-nitrogen balance and cytokinin informs growth control networks controlling shoot-root mass ratios. Phosphate starvation responses follow demand determined by growth activity and not simply availability in the environment.

4.2 Introduction
The ability of plants to respond appropriately to nutrient availability is of fundamental importance for their adaptation to the environment (Lopez-Bucio et al., 2003). Plants require adequate and balanced quantities of mineral nutrients for optimal growth, but such conditions are rarely found in nature. Generally, carbon, nitrogen and inorganic Pi availability constrain plant growth and affect growth patterns (Marschner, 1995). These nutrients trigger and modify molecular mechanisms that change cell division and cell differentiation within the whole plants and have a profound impact on plant development processes. Despite their fundamental importance for growth and growth control, still very little is known at
the molecular level about plant growth control pathways and how they perceive and process environmental signals.

Carbon, phosphate and nitrogen fluxes are coupled through key reactions of primary metabolism, which requires cytoplasmic homeostasis for optimal operation. To maintain homeostasis, while both cellular demand and systemic supplies of macro-nutrients dynamically change, regulation occurs at several levels: control of metabolite flux and allocation, regulation of gene expression, control of metabolite and mineral nutrient re-mobilisation and transport within and between tissues of the plant, and overall growth control of the plant. Very little is still known about the mechanisms regulating these processes. Little is known about how metabolic homeostasis, which underpins plant growth and development, is regulated at the systemic level and how the different levels of control interact with each other.

4.2.1 Metabolic homeostasis

Photosynthesis, intermediate energy metabolism and the synthesis of membrane lipids and nucleic acids all have high Pi requirements. Coupling of phosphate and carbon flux in metabolism occurs at three important control points: during glycolysis, the uptake of Pi into plastids in exchange for carbon skeletons, and during assimilate partitioning into sucrose and starch (Plaxton and Carswell, 1999). Carbon and nitrogen metabolism are also tightly linked to balance the provision of carbon skeletons with the availability of assimilated nitrogen for amino acid and nucleoside biosynthesis, to ensure the presence of adequate reducing equivalents required for N-assimilation, and to sequester ammonium lost in photorespiration (Coruzzi and Zhou, 2001; Stitt et al., 2002).

Metabolic flux can be controlled directly by feed-back or feed-forward allosteric regulation of key pathway enzymes, and this mode of regulation contributes to the stability of the metabolic grid. However, while such recursive regulatory systems involving feed-back or feed-forward control are well suited to maintain homeostasis, they are less well suited to mediate adaptation to altered environmental conditions, which requires the input of signals responsive to changed external conditions.
External cues can directly or indirectly affect metabolism, mediated by signalling metabolites that directly affect enzyme activity or by altered expression profiles of biosynthetic genes. For example, the abundance of the signalling metabolite fructose 2,6-bisphosphate, which regulates carbon partitioning between sucrose and starch (Draborg et al., 2001), responds to the nitrogen and carbon nutritional status of the plant (Stitt, 1990; Kulma et al., 2004). Furthermore, when plants are Pi-starved, a battery of genes collectively thought to increase the ability to assimilate phosphate as well as to reduce internal demand are induced with different kinetics (Hammond et al., 2003; Wu et al., 2003). Enhanced Pi uptake is mediated by changes to root and root hair growth that increase surface area (Bates and Lynch, 1996; Ma et al., 2001; Williamson et al., 2001). Moreover, the secretion of organic acids, nucleases and phosphatases increases Pi mobility in the soil and liberates Pi from organically bound phosphate (Abel et al., 2002). Reduction of demand is promoted by the substitution of phospholipids with sulfo- and galactolipids in membranes (Hartel and Benning, 2000; Hartel et al., 2000; Yu et al., 2002), and the induction of metabolic shunts that reduce the overall steady-state cellular requirement for Pi (Plaxton and Carswell, 1999), thereby freeing up Pi for irreplaceable functions. Furthermore, changes in Pi or N availability affect the expression of sugar-regulated genes (Berger et al., 1995; Coruzzi and Zhou, 2001; Stitt et al., 2002).

Pi-starvation also elicits changes to resource allocation and nutrient transport (Jeschke et al., 1997). Re-distribution of nutrients to sink tissues is required to maintain cell growth and metabolism in meristems (Marschner, 1995). For example, in Pi-replete plants, large amounts of Pi are stored in the vacuoles of shoot tissues (Bieleski, 1973; Mimura, 1999). Root to shoot Pi transport in Arabidopsis requires the PHO1 gene (Poirier et al., 1991). Conversely, Pi-starvation increases transport from shoots to roots, which is impaired in the Arabidopsis pho2 mutant, which accumulates Pi in shoot tissues (Dong et al., 1998). A shoot-derived signal distinct from Pi has been proposed to coordinate whole-plant, systemic Pi homeostasis (Liu et al., 1998a; Burleigh and Harrison, 1999). The central role that inter-organ Pi transport plays in balancing whole-plant Pi budgets indicates that all aspects of Pi starvation responses must be analyzed together to understand how plants respond to altered environmental conditions at a systems level.
4.2.2 Growth responses to altered Pi availability

Changes in mineral nutrient availability also affect plant growth patterns and their magnitude. Pi is tightly bound to soil minerals, and therefore, mobility within soils is very low (Tinker and Nye, 2000). As a result, Pi distribution within soils is highly heterogeneous, even at very small spatial scales (Strawn et al., 2002). Heterogeneously distributed mineral nutrients with low mobility, such as phosphate or iron, are only acquired efficiently by root systems with large surface areas, in which the root surface is brought in direct contact with the soil (Lynch, 1995). In contrast, mineral nutrients with high mobility, such as nitrate, will accumulate as solutes over water-impermeable strata. Root growth patterns observed in response to either limiting phosphate or nitrate reflect their different mobility in the soil: Pi-starvation stimulates net growth of the root system at the expense of the shoot and enhances formation of lateral roots and their growth versus primary root growth (Williamson et al., 2001; Linkohr et al., 2002; Lopez-Bucio et al., 2003). In contrast, in limiting nitrate, root apical growth is promoted. Such specific growth responses suggest that nutrient signalling pathways are coupled to overall plant growth control networks to coordinately regulate plant growth and metabolic responses to altered nutrient availability.

4.2.3 Nutrient signalling and growth control

It is likely that phosphate and nitrate are perceived directly, as their incorporation into metabolites is not required for the activation of cognate gene expression responses (Carswell et al., 1997; Scheible et al., 1997), while carbon-status sensing is more complex and depends both on signalling and metabolism (Coruzzi and Zhou, 2001). However, the molecules required for perception of these mineral nutrients and the molecular identity of further initial components of nutrient sensing and growth control pathways are still unknown in plants.

Recent experiments point to an important role for cytokinin in the coupling of nutrient budgets to plant growth responses. Cytokinin accumulates in response to nitrate treatments (Salama and Wareing, 1979), likely mediated by the induction of the iso-pentenyl-transferase (IPT3) gene, which is rate-limiting for cytokinin
biosynthesis (Miyawaki et al., 2004). In *Arabidopsis* and *Zea mays*, some response regulators involved in cytokinin signalling are induced by nitrate (Sakakibara et al., 1998; Kiba et al., 1999). Furthermore, cytokinin negatively regulates phosphate starvation responses (Martin et al., 2000; Franco-Zorrilla et al., 2002). Further to specific roles in mineral nutrient signalling, cytokinin has global effects on the regulation of root and shoot growth. Cytokinin stimulates the activity of shoot meristems, but inhibits root meristems (Werner and Kinne, 2001; Werner et al., 2003), and hence, cytokinin levels control shoot-root mass ratios. Cytokinin, together with auxin, is required to stimulate entry into and progression through the cell division cycle (Skoog and Miller, 1957; Redig et al., 1996; Zhang et al., 1996; Riou-Khamlichi et al., 1999; Dewitte et al., 2003). Furthermore, cytokinin stimulates rDNA transcription, which is required for the enhanced production of ribosomes associated with cell growth (Gaudino and Pikaard, 1997). Together, these observations suggest a mechanistic basis for cytokinin effects on growth control.

The involvement of different nutrients as well as more global regulatory systems of plant growth responding to nutrient deficiencies or imbalance, raises questions as to how these regulatory levels interact and whether they are hierarchically organized to control plant growth and development. Therefore, we examined the relationship between C and N availability and phosphate nutrition by assessing their effects on the root/shoot mass and the regulation of Pi starvation responses. We evaluated gene expression at the transcriptional level and growth responses as well as Pi levels in root and shoot organs. Our results suggest that the perception of C and N macro-nutrients informs plant growth control mechanisms that in turn control phosphate-starvation responses.
4.3 Results

4.3.1 Phosphate starvation-dependent plant growth and molecular responses are modified by increased carbon availability

In Chapter 3, I showed that molecular marker gene expression responded to the perceived Pi condition in plants (Fig. 3.1). This provided us with efficient tools to examine the relationship between root growth, phosphate starvation-responsive gene induction and the availability of nutrients under different Pi conditions. As we were focussing on the time of onset and the magnitude of phosphate starvation-responsive gene induction, longer time courses were used to show the significant differences.

First, we examined the induction of Pi starvation-inducible gene expression under -Pi or +Pi conditions. In 2 to 6 days, after Arabidopsis seedlings had been transferred to -Pi media, the expressions of the Pi responsive genes (which we discussed before) were several-fold higher than in +Pi media (data not shown). Interestingly, MGD3 gene expression showed the largest magnitude of induction at comparable time-points. This result was recently also confirmed by micro-array analysis: it was also shown that genes coding for enzymes involved in galacto-lipid synthesis were strongly up-regulated during Pi deprivation in Arabidopsis (Julie et al., 2005).

We then compared the Pi starvation responses of Arabidopsis seedlings growing in 0.5x Johnson media (Johnson et al., 1957), which has lower solute concentration, with that of the seedlings in 0.5x MS media (Murashige and Skoog, 1962). In 0.5x Johnson media, after the withdrawal of Pi, MGD3 RNA abundance in Pi-depleted seedlings was more than 10-fold higher than that of the seedlings in 0.5x MS media (data not shown). So in all further experiments, 0.5x Johnson media was used to analyse the Pi starvation responses.

Having established suitable experimental conditions, different concentrations of sucrose were used to examine how carbon availability modifies phosphate starvation responses. In an experiment, after transfer to -Pi 0.5x Johnson media, both in the shoots and in roots, MGD3 gene expression was remarkably induced up to six days and in the same media but with 2% sucrose supplements, this induction was even higher (Fig. 4.1 A: Shoots, B: Roots).
Phosphate starvation-induced $MGD3$ expression is enhanced by carbon supplements in (A) shoots and (B) roots. *Arabidopsis* seedlings were grown hydroponically in the presence of phosphate for 13 days and then transferred to Pi-deplete media with 0.3% sucrose or 2% sucrose. $MGD3$ gene expression was monitored by Real time RT-PCR. Error bars show SEM from a representative experiment.

To assess how sugars might affect Pi signalling within the plant under Pi-depleted conditions, we examined Pi starvation responses in different carbon sources. Plants were grown hydroponically in the presence of Sucrose (Suc), glucose (Glu) or 3-o-methyl glucose (3-OMG), a glucose analogue that triggers sugar signalling but does not participate in metabolism (Smeekens, 2000). The addition of 2% Suc or 2% Glu to the medium, but not of 2% 3-OMG, significantly enhanced $MGD3$ RNA abundance in response to Pi starvation in roots compared with 0.3% sucrose (Fig. 4.2A). During the time course, Pi levels in roots declined from an initial concentration of ~15 nmol to ~5 nmol Pi per mg fresh weight (FW) (Fig. 4.2B), with the initial rate of decline slightly higher in plants grown in 2% Suc or Glu without additional Pi, when compared to controls (Fig. 4.2B). Root Pi levels did not decline substantially when plants were grown in the presence of 3-OMG. This suggests that Pi requirements for carbon metabolism have been enhanced by additional carbon supply.

In contrast to root Pi levels, plant growth was significantly affected by carbon availability. Phosphate starvation strongly promoted shoot growth, while slightly inhibiting root growth in the absence of carbon-supplements (Fig. 4.2C). However, the addition of 2% Suc or Glu to Pi-deplete media specifically promoted root growth, while inhibiting shoot growth (Fig. 4.2D). Root and shoot growth were inhibited by 2% 3-OMG in the media (Fig. 4.2D). Taken together, these data indicated that 2%
Suc or Glu promoted root growth at the cost of shoot growth, indicating that carbon-controlled growth responses dominate over Pi limitation-regulated growth. Pi levels in roots and shoots (data not shown) declined to similar concentrations in plants grown in the presence or absence of 2% sugar, while root mass continued to increase. Therefore, it also can be concluded that enhanced carbon availability promotes Pi transport from shoots to roots or alternatively, carbon-supplements make Pi utilization in roots much more efficient.

Fig. 4.2 Sugar enhancement of phosphate starvation responses requires carbon metabolism. All data are from the same, representative experiment.

A: Phosphate starvation-induced MGD3 expression is enhanced by sucrose or glucose, but not by the non-metabolizable sugar 3-o-methyl glucose (3-OMG). Arabidopsis seedlings were grown hydroponically in the presence of phosphate for 13 days and then transferred to Pi-deplete media with or without supplements. MGD3 gene expression was monitored by Real-time RT-PCR; error bars show SEM. B: Root phosphate concentration declines to similar levels in hydroponically grown plants in the presence or absence of metabolizable sugar supplements. Arabidopsis seedlings were grown as in A; error bars show SEM. C: Shoot and root organ fresh weights are differentially affected by Pi starvation. D: Root growth is specifically stimulated, while shoot growth is inhibited, when Arabidopsis seedlings grow in Pi-depleted conditions in the presence of sugar supplements. All the shoots and roots masses in different sugar condition are compared with the shoots and roots masses under normal –Pi condition.
4.3.2 Phosphate starvation responses are suppressed by nitrogen availability

Carbon, phosphate and nitrogen are the three most important macro-elements for all life forms. Their metabolism is tightly linked in plants and therefore, we examined phosphate starvation responses under varying levels of nitrogen availability in the medium. Moreover, as cytokinins have been implicated in both phosphate starvation responses (Martin et al., 2000; Franco-Zorrilla et al., 2002), and nitrate signalling (Sakakibara et al., 1998; Miyawaki et al., 2004), the potential interactions between these different cues were examined.

When 0.5x Johnson medium (8mM total N) was supplemented with 10mM N (nitrate and ammonium in the same proportions as in Johnson medium), a dramatic reduction in the magnitude of Pi starvation-induced \textit{MGD3} expression was observed in roots after withdrawal of phosphate from the medium (Fig 4.3B). The addition of kinetin to Pi-deplete medium resulted in a suppression of root phosphate starvation responses, in a dose-dependent manner (Fig 4.3B). The suppression of phosphate starvation-induced gene expression in roots was confirmed in the -Pi media with nitrate or ammonium supplements respectively. This suppression was also enhanced when both N and kinetin were added (data not shown). In contrast, phosphate starvation-induced \textit{MGD3} expression in shoots was only modestly suppressed by elevated N early in the time course, and enhanced by kinetin treatments (Fig. 4.3A). We concluded that Pi-starvation responses are distinct in roots and shoots in the presence of increased N or kinetin availability.
**Fig. 4.3** Increased abundance of nitrogen or kinetin represses phosphate starvation responses in roots, but enhances these in shoots. Seedlings were grown hydroponically in the presence of phosphate for 13 days and then transferred to Pi-deplete media with the supplements indicated or to Pi-replete medium.

A: Phosphate starvation-induced *MGD3* gene expression was enhanced in shoots, monitored by Real time RT-PCR; error bars show SEM.

B: Phosphate starvation-induced *MGD3* gene expression was repressed in roots, monitored by Real time RT-PCR; error bars show SEM.

To distinguish whether nitrate perception or nitrogen metabolism were required for the observed effects on phosphate starvation responses, we used the nitrate reductase (*NIA*) double mutant G'4-3, which has ~1% residual NR activity in roots (Wilkinson and Crawford, 1993; Lejay et al., 1999). *nia1, nia2* double mutants still respond to external nitrate, indicating that nitrate sensing is distinct from nitrate metabolism (Lejay et al., 1999; Wang et al., 2004a). Wild type and mutant plants were grown hydroponically and subjected to Pi starvation with additional nitrate, ammonium or both. In the resulting time course, *MGD3* RNA abundance in roots of the *nia1, nia2* double mutant responded very similarly to the wild type, and specifically still suppressed root Pi-starvation responses when supplemented with nitrate or ammonium alone (*Fig. 4.4*). This indicated that nitrogen perception was required to suppress Pi starvation responses in roots. We cannot be completely sure that only nitrogen perception was involved, because there was still ~1% NR activity in roots. The fact that provision of ammoniums alone suppresses *MGD3* RNA induction under Pi limiting conditions also indicates that nitrogen metabolism can suppress Pi starvation responses.
Increased abundance of nitrogen represses phosphate starvation responses in nia1, nia2 double mutant in roots.

Seedlings were grown as before. MGD3 gene expression was monitored by Real time RT-PCR; error bars show SEM.

As Nitrogen and Kinetin affect Pi starvation responses on the molecular level in a similar way, we then assessed whether the nitrogen cue was acting through the cytokinin-signalling pathway to control the magnitude of phosphate starvation responses. Therefore, we compared starvation-induced MGD3 expression in wild-type, and in two alleles of the cre1 mutant (cre1-2 and cre1-4) (Inoue et al., 2001). CRE1 encodes a cytokinin receptor that belongs to a small gene family and the two mutants display reduced sensitivity towards cytokinin (Inoue et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004). After transferred seedlings to media without Pi, starvation-induced MGD3 expression in roots was modestly elevated in cre1-2 and cre1-4 seedlings, when compared to the wild type and the phr1-1 mutant (Fig. 4.5B). This indicated that endogenous levels of cytokinin suffice to suppress Pi-starvation gene expression responses. However, when grown in 0.5x Johnson media with the addition of either Nitrogen or Kinetin, cre1-4 mutants still responded to these conditions, showing that single mutants of the cytokinin receptor gene family are still responsive to the plant growth regulator (Fig. 4.5A). These results confirmed that nitrogen or cytokinin suppress Pi starvation responses by indirectly controlling plant metabolism, but not by one signalling pathway.
Nitrogen or kinetin represses phosphate starvation responses in roots of cre1-4 mutant. MGD3 gene expression was monitored by Real time RT-PCR; error bars shown SEM.

Phosphate starvation-induced MGD3 expression in different mutants. Seedlings were grown as before. MGD3 gene expression was monitored by Real time RT-PCR; error bars show SEM.

4.3.3 High nitrogen or cytokinin affect on shoot and root growth differentially

Cytokinins affect growth of the plant body in Arabidopsis by enhancing the activity of shoot, while suppressing the activity of root meristems (Skoog and Miller, 1957; Williamson et al., 2001). Hence, the influence of cytokinin on Pi starvation signalling could be due to a change in the growth rate of roots and shoots or, alternatively, could be caused by a role of cytokinin responsive transcription factors in the expression of Pi starvation-induced genes. To distinguish between these possibilities, we measured plant growth rate and Pi concentration in shoot and root tissues of plants grown within different levels of nitrogen and kinetin.

Following the withdrawal of Pi from the medium, the Pi concentration in roots declined in the first four days of the time course and then stabilized at a concentration of ~5nmol/mg fresh weight (FW) (Fig. 4.6A). Addition of N to the medium delayed the decline in Pi concentration, while addition of kinetin or both N and kinetin resulted in root Pi concentrations that were higher than in the control with 1.0mM Pi in the medium (Fig. 4.6A). In contrast, the Pi concentration in shoot tissues was reduced by the addition of N, and more markedly reduced by the addition of Kinetin or both N and Kinetin (Fig. 4.6B). These effects of N and Kinetin on root and shoot phosphate content are not specific to Pi-deplete growth conditions, as a similar pattern of tissue Pi content is observed under Pi-replete growth conditions.
(Fig. 4.6 G, H). This suggests that N, or Kinetin, trigger the changes in root, shoot growth and Pi content, which dominate over Pi starvation responses, when C and N availability are more balanced.

**Fig. 4.6** Nitrogen or kinetin regulates organ steady-state phosphate levels and shoot-root mass ratios in Pi-deplete plants. Seedlings were grown as before.
A, B: Tissue phosphate levels were determined from aqueous extracts of roots (A) and shoots (B), respectively; error bars shown SEM. Phosphate levels decline less in roots with nitrogen or kinetin supplements, while they decline more rapidly in the corresponding shoots. C, D: Tissue fresh weight was determined from roots (C) and shoots (D). Growth is suppressed in roots with nitrogen or kinetin supplements, while it is slightly enhanced in the corresponding shoots. E, F: Tissue fresh weight was determined from roots (E) and shoots (F). G, H: Tissue phosphate levels were determined from aqueous extracts of roots (G) and shoots (H), respectively; error bars shown SEM. Phosphate levels decline less in roots with nitrogen or kinetin supplements, but decline more rapidly in the corresponding shoots.

The observed differences in tissue Pi concentration might reflect differences in the mobilization of Pi between tissues, but could simply also be the results of differential growth of roots and shoots under these conditions. The increase in fresh weight of roots observed after transfer to media without Pi addition was markedly lower in those plants grown with additional N, kinetin or both (Fig. 4.6C), while in shoot tissues the opposite was observed (Fig. 4.6D). These data indicated that at least part of the increase in Pi concentration we observed in Pi-starved root tissues grown in the presence of additional N, kinetin or both was attributable to a relative decrease in root mass, whereas in shoots, a decrease in Pi concentration correlated with an increase in tissue mass.

4.3.4 High nitrogen or cytokinin suppress the phosphate uptake in root

To better understand the effects of nitrate or cytokinin on Pi starvation responses, we used $^{32}$Pi on Pi-deplete plants to check the plant Pi uptake rate from the Pi-depleted media with different N or cytokinin supplements.

When Pi starved plants were provided with $^{32}$Pi, there was a very high rate of Pi uptake. After supplying $^{32}$Pi to +Pi plants, from the image we can see, the radioactivity was not as strong as Pi-depleted plants and $^{32}$Pi has first been stored in the shoots as a reservoir (Fig 4.7). In Pi depleted media with additional nitrate or Kinetin, the plant uptake rate of $^{32}$Pi was not as strong as in Pi-deplete media without N or Kinetin, both in short and long time courses (Fig 4.7, Fig 4.8). These results confirmed that the nitrogen or cytokinin had negative effects on the uptake rate of Pi from the environments. Also as we expected, supplement of Kinetin can affect Pi distribution between shoots and roots (Fig 4.8). Taken together with our previous results, under high nitrogen or cytokinin condition, the suppression of Pi starvation...
response gene expression caused by the plant root growth has been restrained and a higher amount of Pi has been accumulated in the roots compared with lower nitrogen or cytokinin conditions.

Fig. 4.7 Nitrogen or kinetin affects phosphate uptake rate in phosphate deplete plants in short-time course.

Left: Phosphate image pictures shown the radioactivity level in plant roots; A: -Pi 0.5x Johnson media, B: 10mM Pi 0.5x Johnson media, C: 10uM Kinetin —Pi 0.5x Johnson media, D: 20mM KNO₃ —Pi 0.5x Johnson media.

Right: Quantitative analysis of radioactivity in roots. Seedlings were grown hydroponically in the Pi-deplete media for 6 days and then transferred to the same media with the supplements indicated or to Pi-replete medium. Every single time-point was 1.0cm root's average radioactivity from two individual plants. Error bars show SEM. Left and right were two independent experiments.

Fig. 4.8 Nitrogen or kinetin affects phosphate uptake rate in Pi-deplete plants in long-time course.

Left: Phosphate image pictures to show the uptake rate of 32-P. A: -Pi 0.5x Johnson media, B: 10uM Kinetin —Pi 0.5x Johnson media, C: 0.5x Johnson media with 2.5mM Pi. Right: Quantitative analysis of total 32-P in shoot and root. Error bars show SEM. Seedlings were grown hydroponically in the Pi-deplete media for 6
days and then transferred to the same media with the indicated supplements or to Pi-replete medium.

4.3.5 Different phosphate starvation responses under nitrogen or carbon condition are not mediated by osmotic responses

From previous sections, we observed under Pi starvation conditions changes in carbon or nitrogen availability affect Pi starvation-induced gene expression and plant growth respectively. To distinguish whether the effects on Pi starvation-induced gene expression are caused specifically by differential availability of nutrients or by their effects on growth, we altered the osmotic conditions in the medium. High osmotic levels are known to negatively affect plant growth without changing carbon or nitrogen availability (Hasegawa et al., 2000).

Following withdrawn of Pi from the media, two osmotic controls were used on Pi-deplete seedlings, Mannitol and KC1. In plant roots, Pi starvation responses were also suppressed under these conditions (Fig. 4.9A). These results showed that high osmotic levels of solutes reduce phosphate starvation responses.

To examine if plant growth was affected by these conditions, we examined root growth. The data showed that root growth was repressed by provision of mannitol. But this repression of root growth did not occur by addition of KCl (Fig. 4.9B). We then measured the Pi concentration in shoots and roots in plants grown under these conditions. Unlike the results under additional N or cytokinin condition, the Pi concentration in Pi-deplete plants with osmotic molecules declined in the first four days and then stabilized just as wild-type control plants, both in shoots and roots (data not shown). This data suggests the osmotic molecules did not alter the Pi concentration in plants even the plant growth was altered.

From the Mannitol data, it can be concluded that osmotic responses can restrain plant root growth and the limitation of root growth could have negative affects on Pi starvation responses. However, we still have problems to explain KCl results here. One possibility is that high concentration of potassium can increase cell extension but not increase the cell numbers in the roots. Even if the cell numbers in the roots have been decreased in plant roots which caused by osmotic responses, the totally root length is still increased under high potassium condition by increasing the cell
size. Taken together, the conclusion is the Pi starvation responses is regulated by the alterations of plant growth pattern, which are not directly mediated by osmotic responses.

Fig. 4.9 Osmotic responses restrain phosphate starvation responses and plant growth in plant. 1.06% mannitol has been used as the same number as 2% sucrose.

A: Osmotic responses repressed phosphate starvation responses. B: Osmotic responses restrain plant root growth. All the root length data compared with the root length in -Pi media. Seedlings were grown on the vertical plates in the corresponding media and measured primary root length at 7th and 13th day. Error bars shown SEM.
4.4 Discussion

By changing macro-element availability and using a Pi starvation responsive marker gene to Arabidopsis seedlings, we examined the interactions between C, N and Pi nutrient availability and the relationship of phosphate starvation responses to plant growth. Under Pi starvation conditions, increased carbon availability enhances plant root growth and restrains shoot growth, whilst elevating Pi starvation induced gene expression. In contrast, increased nitrogen availability, or Kinetin supplements, strongly suppresses Pi starvation induced gene expression in roots, but enhances expression of these genes in shoots. Meanwhile, high N or cytokinin conditions inhibit root growth and slightly promote shoot growth, independent of Pi availability in plant. These changes in shoot and root biomass correlate with elevated root and decreased shoot Pi concentration. Taken together, our results suggest that the magnitude of Pi-starvation responses is controlled by plant organ growth. We propose that by differential control of shoot and root organ growth C or N metabolisms affects the magnitude of Pi starvation signalling in plant.

4.4.1 Carbon metabolism

It is well known that inorganic Pi plays a central role in plant metabolism both as a substrate for photo-phosphorylation and ATP generation through glycolysis and respiration as well as a key component for the export of carbon from chloroplasts during photosynthesis. Therefore, it is not surprising that supply of sugars enhances Pi starvation responses in the plant (Fig. 4.1). This suggests that Pi requirements for carbon metabolism are enhanced by additional carbon supply. This was also confirmed by the control experiment with 3-OMG (Fig. 4.2A), a glucose analogue that triggers sugar signalling but does not participate in metabolism (Smeekens, 2000). Although addition of sugars accelerates the initial reduction in root Pi concentration in response to Pi depletion, our results show that final root Pi levels in carbon-supplemented plants can not clearly be distinguished from those without sugar supplements (Fig. 4.2B), despite starvation responses being much enhanced in the former. Moreover, in Pi depleted plants, roots grow faster with carbon supplements than those without additional sugar (Fig. 4.2D). Plant shoot growth was slightly inhibited by Pi supplement without carbon supplements (Fig. 4.2C) and the
leaf senescence rate was increased in these conditions (data not shown). Together, these results indicate that residual Pi must be used much more efficiently within the cells to accelerate root growth and mobile Pi can be re-located or re-distributed between shoot and root. This could be a consequence of the enhanced Pi starvation responses, some of which reduce the metabolic requirement for Pi (Plaxton and Carswell, 1999; Hartel et al., 2000).

Taken together, we propose that sugar supplements enhance Pi starvation responses by increasing Pi demand by the whole plant. The effect of sugar supplements, even under Pi limiting conditions, indicates that metabolic control is not exerted by the minimum nutrient at any given time (van der Ploeg et al., 1999), but rather in a hierarchical fashion, such as that carbon metabolism dominates over Pi metabolism.

4.4.2 Nitrogen metabolism and cytokinin

After increasing the levels of nitrate, cytokinin or ammonium available to Arabidopsis seedlings, we observed that Pi starvation responses were suppressed in roots in a dose-dependent manner (Fig. 4.3B). In contrast, Pi starvation responses in shoots were only moderately suppressed by elevated nitrogen availability early in the time course, and were enhanced by kinetin treatments (Fig. 4.3A). In previous studies, it was reported that Pi starvation responses were suppressed by cytokinin treatments in a wild-type allele of the cytokinin receptor CRE1 (Martin et al., 2000; Franco-Zorrilla et al., 2002). These results were also ascribed to the affects of cytokinin on long-distance systemic responses to Pi starvation. However, changes in organ growth and Pi levels in response to kinetin treatments were not reported in those studies.

Endogenous cytokinin levels are reduced in Pi starved plants (Salama and Wareing, 1979), while nitrogen supplements elevate cytokinin levels and induce expression of cytokinin biosynthetic genes (Miyawaki et al., 2004). Here, we have shown that increased nitrogen availability in the growth media mimics the effects of kinetin treatments, and that both affect shoot-root growth ratios and organ Pi content (Fig. 4.6). Kinetin, or high nitrogen treatments, suppress root growth, enhance shoot growth and increase Pi content in roots while decreasing Pi content in shoot in both Pi-deplete or Pi-replete media (Fig. 4.6).
Therefore, we propose that suppression of Pi starvation induced gene expression in roots in the presence of kinetin (Martin et al., 2000; Franco-Zorrilla et al., 2002), or nitrogen is likely caused by altered control of whole-plant growth patterns and unlikely to be the result of a direct effect of kinetin, or a high nitrogen supply. It is interesting to see that the effects of kinetin are stronger and more durable than those observed with nitrate or ammonium supplements, possibly because nitrogen is consumed in metabolism or sequestered, so as to render it less effective. Furthermore, the effects on gene expression levels observed in experiments with carbon and nitrogen supplements under Pi starvation conditions support the notion that Pi levels that cue gene expression responses are sensed in the cytoplasm (Kock et al., 1998).

4.4.3 Growth control

Although not much is yet known about plant growth control machinery at the molecular level, it is clear from our data increased carbon availability enhances plant root growth and restrains shoot growth. Meanwhile, high N or cytokinin conditions inhibit root growth and slightly promote shoot growth. These changes in shoot and root growth correspondingly alter the Pi starvation responses in plant, independent of Pi status. Taken together, we conclude that carbon, nitrogen or cytokinin metabolisms govern plant organ growth and the growth rate controls the demand for phosphate and the magnitude of Pi-starvation responses.

<table>
<thead>
<tr>
<th>Plant organ</th>
<th>Growth rate</th>
<th>Pi starvation responses</th>
<th>Pi concentration</th>
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<tr>
<td><strong>High Carbon availability</strong></td>
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<tr>
<td>Shoots</td>
<td>-</td>
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| **High Nitrogen/Cytokinin availability** |     |                         |                  |
| Shoots          | +           | +                       | -                |
| Roots           | -           | -                       | +                |

Table 4.1 Model of how carbon and nitrogen availability dominate *Arabidopsis* phosphate starvation responses and their relationship with plant growth.
5 Genetic analysis of phosphate signalling in *Arabidopsis*

5.1 Summary

To identify genes involved in Pi signalling, a genetic screen with a Pi starvation-responsive reporter line was performed. By providing Pi to Pi-starved mutants, it is possible to find the mutants which negative regulate Pi signalling pathways. A homozygous line carrying the *At4:: LUC* reporter was generated and mutagenized by $\gamma$-ray mutagenesis. 6,000 $M_1$ seedlings were planted and 2,000 $M_2$ pools screened. A candidate mutant (L809) was identified and initial analysis showed it is likely to be a signalling mutant. Two further binary vectors, *Phit1;1::LUC* and *MGD3::LUC* were constructed and transformed into *Arabidopsis* plant (Col-O) for use in future screens.

5.2 Introduction

To enhance our knowledge about molecular processes associated with Pi deficiency and also facilitate the identification of key molecular components involved in the initial steps of the Pi signalling pathway, a forward genetic screen was carried out. The firefly luciferase gene (*LUC*) was used as an ideal reporter under control of Pi-responsive promoters. The advantages of luciferase are that it has a short half-life in planta and its activity in whole plants can be assayed by imaging for its luminescent reaction using a sensitive camera (Millar et al., 1992). Luciferase activity is usually measured by photon-counting, intensified charged-couple device (CCD) cameras. Intensified CCD cameras allow for the detection of extremely low light levels and are therefore well suited for monitoring low levels of luciferase activity (Michelet and Chua, 1996).

Most of the *PSI* genes we analysed are expressed in plant roots (Muchhal et al., 1996; Burleigh and Harrison, 1999; Awai et al., 2001). When reporter lines were grown in Pi-deplete conditions, the *LUC* gene was highly activated in the roots. After addition of luciferin, the luciferase activity can be captured by CCD camera. Then provision of Pi or Phi to these seedlings can help us to monitor the kinetics of promoter-dependent luciferase gene expression in vivo. As the additional Pi or Phi can down-regulate the *PSI* genes expression and correspond to the luciferase
activities, the kinetics of luciferase gene expression shows the immediate-early response of Pi signalling in plant roots. The mutants, which were shown the altered kinetics, would be selected as our candidates. In the candidate mutants, if a real deviation of luciferase activities compared with the expression pattern in wild-type can be detected, it suggests that the gene which lost its function in the candidate mutant is involved the early responses of Pi signalling pathway and this mutant is a phosphate signalling mutant. By using a genetic screen based on the mutagenesis of these reporter lines, it will help us to identify the key elements including phosphate receptor, sensor or important regulators in the early-responses of Pi signalling pathway in plant.

5.3 Results

5.3.1 Generation of Pht1;1 or MGD3 promoter-luciferase gene constructs

Based on the publicly available Arabidopsis genomic sequence database (http://arabidopsis.org/index.jsp), primers were designed and PCR amplifications were performed to amplify fragments comprising the PHT1;1 and MGD3 promoters respectively. After their sequences were confirmed by DNA sequencing, these promoters were fused to the LUC reporter gene (LUC+, purchased from Promega, but modified to introduce a KpnI and SaeI site immediately downstream of the LUC stop codon) and inserted into the plant transformation vector. Two Pht1;1 promoter constructs were made, one with the native promoter, one which carries an Ω enhancer between the promoter and the luciferase gene, which elevates luciferase gene translation, because it can function as an internal ribosome entry site (IRES) (Hellen and Sarnow, 2001). The final constructs were transformed into Arabidopsis plants (Columbia background) by Agrobacterium-mediated transformation. Homozygous PHT1;1 and MGD3 promoter (Ω): Luciferase reporter lines are not included in the work of this thesis but these reporter lines will be used in future to screen for Pi signalling mutants.

5.3.2 Characterization of At4::luciferase reporter lines

The same approach was used to generate At4::LUC transformed plants (detail see Whyte, 2004). After selection for homozygous At4::LUC reporter line, this reporter
line was characterized to examine the down-regulation of luciferase gene expression by provision of Pi.

The seedlings were first grown vertically on plates with 0.5x MS media, and were then transferred to -Pi media to induce Pi starvation. The Pi-deplete seedlings were pre-sprayed with luciferin three times at 6 hours intervals. As luciferase protein is stable in the absence of luciferin, this was required to reduce background activity due to the accumulation of luciferase over time. Without such treatment, luciferase activity is not proportional to the activity of the promoter used to express LUC (van Leeuwen et al., 2000). 10mM Pi was then sprayed on to seedling roots and luciferase gene expression was monitored by a CCD-camera (Hamamatsu Digital camera C9100). Images obtained in different time courses were analyzed and quantified by using Simple PCI 5.2.1.

![Fig. 5.1 At4::LUC reporter line responds to Pi supply in Pi-deplete seedlings.](image)

As shown in Fig. 5.1, after Pi-starved At4::LUC seedlings were sprayed with 10mM Pi, luciferase activity could be clearly seen to diminish in roots in the following time course, disappearing within 6-8 hours after provision of Pi. These data are quite
similar to previous RNA down-regulation results by providing Pi in Pi-deplete cells and plants (Fig 3.1). This verifies the responsiveness of our reporter line to Pi and illustrates the decay of luciferase activity in \textit{At4::LUC} reporter line. The same plants would be used in genetic screens to identify signalling components \textit{in vivo}. Fig. 5.2 shows the luciferase gene expression in \textit{35S::LUC} seedlings as control.

![Image](image.png)

\textbf{Fig. 5.2} \textit{35S::LUC} reporter line does not respond to Pi supply in Pi-depleted seedlings.

5.3.3 \textit{A γ-ray mutagenesis screen based on the At4::luciferase reporter line}

In order to perform a genetic screen to identify genes involved in the early responses of Pi signaling, it was necessary to first mutagenise these seeds. For this purpose, after mutagenesis Pi has to be provided to all the Pi-deplete seedlings to analyze the kinetics of luciferase gene expression. The mutants showed the altered kinetics would be selected as our candidates.

About 7000 \textit{At4::LUC} reporter line seeds were measured by weighing 100 seeds and multiplying up to calculate the weight of 7000 seeds. The seeds were placed next to a γ-ray source for mutagenesis, giving an absorbed dose of radiation of 300 Grays.
This dosage had been recommended as being optimum for mutagenesis for saturation of the whole genome of *Arabidopsis* and had been used in the lab previously (Gifford, 2004). This original population of mutagenised seeds was designated the M₀ generation.

All M₀ seeds were sown immediately after mutagenesis on to MS agar plates to allow germination to take place. The seedlings from M₀ seeds were named as M₁ population. 14 percent of M₀ seeds were infertile. From this initial M₁ population, 6000 individual M₁ seedlings were transplanted to soil. After eight weeks on soil, seeds from M₁ plants were collected. Seeds were collected from individual M₁ plants. Each M₂ line will consist of plants segregating for homozygous or heterozygous mutants, or wild type at the M₁ mutation sites.

About 15 seeds from each M₁ population were sown individually on vertical plates and the resultant M₂ seedlings of each line were screened. The same protocol as for characterization of the *At4::LUC* reporter line was used to select the candidates. This screen was carried out for 2000 of the M₁ lines. The kinetics of down regulation of luciferase gene expression in response to phosphate perception was tested by provision of 10mM Pi to Pi-deplete plants. In the first pass of screening, we did not quantify the images, as this would have been too much work. However, as there were wild type siblings on each plate as internal control, we could easily compare the kinetics of change in luciferase activity in these and any candidate mutant by eye. Any mutant lines of which showed abnormal kinetics of luciferase gene expression were further examined. After screening on plates, the candidates were transplanted individually to soil to harvest seeds.

123 lines were re-screened in the same M₂ population to confirm the candidates. Of these lines, 56 lines were rejected as their luciferase gene expression kinetics proved to be similar to that of wild type plants. Of the remaining lines, 12 lines contained lethal mutations and did not perform further analysis. The 55 remaining lines were continued to analyse in the M₃ population.

In the M₃ population, 54 lines showed no or weak luciferase activity in Pi-deplete seedlings. One line (809) showed elevated luciferase expression in Pi-deplete plants
Fig. 5.3 At4::LUC reporter line and mutant (809) responds to Pi supply in Pi-deplete seedlings
and slower down-regulation of luciferase gene expression in response to Pi compared to wild type reporter lines (Fig. 5.3).

5.3.4 Initial analysis of candidate mutant

To confirm that this candidate line is a Pi signalling mutant, careful characterization is still required. Using the protocol described above, we quantified luciferase activity in the M3 population of the candidate line (809), shown in Fig. 5.4. In this mutant background, the luciferase activity in Pi-deplete seedlings was down regulated by provision of Pi. However, the velocity and magnitude of the down regulation was attenuated compared to wild type. The difference of luciferase activities in 809.1 and 809.2 plants might be due to other mutations might be existed in this line. Therefore, it is still segregating for these mutations even this mutant is homozygous for one gene underlying the observed phenotype.

![Graph showing luciferase activity](image)

**Fig. 5.4** Quantitative analysis of luciferase gene expression in Wt & mutant (809)

809.1 and 809.2 are individual plants in M3 population of 809 mutants. In each series, the luciferase activities at 0hr time-point have been normalised to 100% separately.

These results of the quantitative analysis suggest that this mutant (809) is involved in Pi signalling. Further analysis by RT-PCR would demonstrate any effects of this mutation on PSI gene expression. Moreover, a map-based cloning approach will identify the gene underlying this mutant and help us to further characterize its function in the early responses of Pi signalling pathway.
6 Conclusion and Discussion

Through this work, we have tried to dissect some of the complicated and difficult aspects of the Pi signalling pathway. These included both the local and systemic Pi signalling pathways as well as the relationships between the phosphate signalling pathway and other nutrient metabolisms. By analyses of immediate-early local and systemic responses to Pi perception, we found very rapid molecular responses to Pi perception, generating the Pi signal in the plant. To characterize the local Pi signalling pathway, which is initiated by Pi perception, we demonstrated that several important steps are involved, including negative regulators, protein kinases and proteolysis. Meanwhile, from the systemic data in my results, we concluded that Pi itself does not appear to be the systemic signal and the \textit{pho2} mutant affects the early responses of Pi signalling. We also used a genetic screen with a Pi starvation responsive reporter line to identify further elements, involved in the early-response of the Pi signalling pathway.

To study the relationships between Pi and other nutrient metabolisms, we used the \textit{MGD3} gene as the molecular marker, because its expression is significantly regulated by Pi status in plant. By combining shoot-root growth ratios and Pi concentration data with the molecular results, we concluded that C and N dominate plant growth and are not dependent on Pi status or the Pi starvation induced growth response.

In the study of the Pi signalling pathway, two factors remain difficult to be resolved. Firstly, there is still a lack of information regarding the important members of the Pi signalling pathway. At present, we do not know what is the Pi sensor or where it is localized or how the Pi transporter proteins are regulated in plant cells. As demonstrated in the protein kinase inhibitor experiments, the lack of knowledge of members, which are involved in the early responses of the Pi signalling pathway, make it difficult to select the inhibitors which directly target this pathway.

The second problem is that plant metabolism is a multi-dimensional process, in which several factors or pathways may work together or with similar functions to contribute to a single result. For example, in the osmotic results, provision of osmotic chemicals can suppress the \textit{MGD3} gene induction in Pi-deplete seedlings.
A similar result is seen with the provision of nitrogen source to Pi-deplete plants (Fig. 4.3 B). They all contribute to the plant root growth and indirectly control Pi starvation gene expression. The interconnecting nature of such aspects of metabolism contributes to the difficulties of resolving Pi signalling questions.

To avoid these difficulties, we have analysed the immediate-early responses of Pi signalling and the systemic signalling which is initiated by Pi perception, therefore avoiding the complications relating to the downstream factors. Also, working on Pi-deplete plants avoids the effects of in vivo Pi in the plant vacuole. These approaches allow us to draw conclusions and build up our model easily from our limited results.

The main aim of my project is to provide a framework of the Pi signalling pathway. For characterization of a signalling pathway, it is always a good idea to start with the framework and then gradually expand. Using the framework I have developed will allow us to identify the key elements involved in the Pi signalling pathway, such as the negative regulators and kinases. We also expect that based on our model, more and more elements will be identified and the whole Pi signalling pathway will become much clearer.
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