A PHOSPHORUS MUTANT OF *ARABIDOPSIS THALIANA*

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Abstract

A phosphorus accumulator mutant of *Arabidopsis* with a recessive mutation at a locus designated *pho2* was previously identified (Delhaize and Randall, 1995). The present project was aimed at further characterisation of the *pho2* mutants by comparison to wild type plants to define the physiological basis of the mutation and to initiate the cloning of the *PHO2* locus.

Accumulation of Pi in leaves of *pho2* was found to reside in the symplast and was not related to Zn-deficiency. A series of ^{32}Pi-labelling experiments under P-sufficient conditions showed that *pho2* mutants had about a two-fold greater Pi uptake rate and transported a greater proportion of the Pi taken up to shoots than wild-type seedlings. When shoots were removed, the difference in uptake rates between the genotypes disappeared, suggesting that the greater Pi uptake by intact *pho2* mutants is due to a greater shoot sink for Pi. *pho2* seedlings could recycle Pi from shoots to roots through the phloem but the proportion of ^{32}Pi translocated to roots was less than half of that found in wild-type plants. The physiology of the *pho2* mutant is consistent with either a block in Pi transport in phloem from shoots to roots or in an inability of shoot cells to regulate internal Pi concentration.

Southern blot analysis of two phosphate transporter genes, *APT1* and *APT2* revealed that the two genes were genetically linked and were both located on chromosome 5. Therefore, mutations in either of these two genes are not responsible for the *pho2* mutation which is located on chromosome 2. Furthermore, expression of these genes was not perturbed in the *pho2* mutant.

Mapping of the *PHO2* gene located it to a sequenced region of about 400 kb between the molecular markers *cop1* and T14G11 on chromosome 2. No obvious candidate for PHO2 was found in the translation products of putative genes in this region. These data, along with information from the *Arabidopsis* genome sequencing, will form the basis for cloning the *PHO2* gene in the future.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree in any university or other tertiary institution and, to the best of my knowledge and brief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopy.

SIGNED: DATE: 8/03/99

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Papers arising:

Dong B, Rengel Z, Delhaize, E. (1998) Uptake and translocation of phosphate by *pho2* mutant and wild-type seedlings of *Arabidopsis thaliana*. Planta. **205:** 251-256.

Dong B, Ryan P, Rengel Z, Delhaize, E. (1999) Changes in the expression of phosphate transporter genes, phosphate uptake and internal phosphate concentrations in *Arabidopsis thaliana* associated with altered phosphate nutrition. Plant Cell Envirion. (accepted).

Smith FW, Ealing PM, Dong B, Delhaize E. (1997) The cloning of two *Arabidopsis* genes belonging to a phosphate transporter family. Plant J. **11:** 83-92.

Delhaize E and Dong B. (1998) Phosphorus mutants of *Arabidopsis*. *In:* Phosphorus in Plant Biology: Regulatory Roles in Molecular, Cellular, Organismic and Ecosystem Processes. Eds, JP Lynch and J Deikman. American Society of Plant Physiologists. pp 242-251.

Abbreviations

ATP	adenosine 5'-triphosphate
bp	base pair
Bq	becquerel
CAPS	cleaved amplified polymorphic sequence
cDNA	complementary DNA
Ci	Curie
cM	centimorgan
°C	degree Celcius
d	day
DNA	deoxyribonucleic acid
DTT	dl-Dithiothreitol
dw	dry weight
EDTA	ethylenediaminetetra acetic acid
EMS	ethyl methanesulfonate
fw	fresh weight
g	gravity
h	hour
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
Km	Michaelis constant
L	litre
MES	2-[N-morpholino] ethanesulfonic acid
min	minute
mL	millilitre
µL	microlitre
P	Phosphorus
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
Pi	inorganic phosphate
RFLP	restriction fragment length polymorphorism
RNA	ribonucleic acid
s	second
SDS	sodium dodecyl sulfate
SSC	sodium chloride (0.15 M) and tri-sodium citrate (0.015 M)

SSLP	simple sequence length polymorphism
TE	Tris (10 mM)-EDTA (1mM)
Tris	tris (hydroxymethyl) aminomethane
w/v	weight/volume
Vmax	maximal velocity
v/v	volume/volume
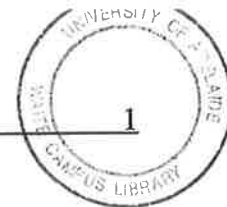
Chapter 1

General introduction

As an essential element for plants, phosphorus (P) is present in nucleic acids, phospholipids, lipopolysaccharides and in many other organic components to participate in important processes, such as energy transport in cell metabolism, carbohydrate synthesis and photosynthesis. Phosphate itself is involved in regulation of enzyme activities via phosphorylations (Marschner, 1995). This chapter begins with a brief discussion about P in the soil and its availability to plants, progressing to how plants manage to take up, transport and allocate Pi (inorganic phosphate) within cells, and finally, describing the molecular and genetic controls of P nutrition in microorganisms and plants.

1.1. Phosphorus in the soil

Agricultural soils generally contain large amounts of total P, but only a small proportion is immediately available for plant uptake. Except near recently applied bands of fertiliser, the Pi concentration in the soil solution is seldom higher than 10 μM, and usually less than 2 μM, while typical concentrations of other mineral nutrients such as K, Ca and Mg are 90, 700 and 1000 μM, respectively (Bieleski, 1973). The major portions of soil P are 1) organic material, including phosphate monoesters, predominantly as inositol hexaphosphate and inositol pentaphosphate, and 2) Pi salts of low solubility representing adsorbed P or precipitated mineral P (Cosgrove, 1967; Newman and Tate, 1980; Richardson, 1994). Soil P minerals are mainly those of Ca, Al and Fe. In alkaline, calcareous soils, Ca phosphates predominate, whereas in acid soils, Fe and Al phosphates are the main P forms (Barber, 1984). Calcium, Fe and Al phosphates are poorly available to plants due to their poor solubility.

Within the pH range of 4.0-8.5 in most soils, the major forms of phosphate in the soil solution are $H_2PO_4^-$ and HPO_4^{2-}. The amount of each form depends on the pH of soil solution, with equal amounts of $H_2PO_4^-$ and HPO_4^{2-} present at near neutral pH. Below this

pH, $H_2PO_4^-$ is the dominant form with evidence indicating that plants primarily take up $H_2PO_4^-$ from the soil solution (Bieleski, 1973).

1.2. Characteristics influencing the ability of plants to access P from soil

There is considerable genetic variation for most morphological and physiological characters associated with the ability of plants to acquire Pi from soils. The mechanisms involved can be mainly identified as influencing surface area for enhancing the uptake of Pi and rhizosphere modifications for improving the acquisition of Pi.

Because of the low mobility of P in soil, an increase in root surface, as achieved through root proliferation or root hair development, leads to greater access of Pi from a given volume of soil. Differences in root hair development and consequently Pi uptake exist among different genotypes within some species (Itoh and Barber, 1983; Green et al., 1991) and selection for root characters has been successful in alfafa, ryegrass and white clover (Pederson et al., 1984; Ennos, 1985; Caradus et al., 1993). The proteaceae species and white lupin develop highly clustered roots under low P supply, often referred to as proteoid roots which are thought to increase Pi uptake (Gardner et al., 1981; Gardner et al., 1983).

Rhizosphere modification by root exudates and microorganisms is also important for plants to access the P sources. Roots exude substances such as amino acids, organic acids, protons, sugar reductants and mucilage which act as chelating agents or alter the soil chemistry (Hedley et al., 1982; Marschner, 1995) to influence nutrient availability in the rhizosphere. Organic acid and acid phosphatases are two types of root exudates that are likely to be directly involved in the P nutrition of plants. Gardner et al. (1983) and Marschner et al. (1995) showed that white lupin (*Lupinus albus*) develops proteoid roots that secrete large quantities of citric acid under low P conditions. Citric acid is highly effective in chelating Ca, Fe and Al and therefore solubilises mineral forms of P in the rhizosphere, allowing its subsequent uptake by plants. The mechanisms used by roots to excrete organic acid are not known but are likely to involve i) regulation of metabolic pathways by internal or external factors for the synthesis of organic acids (Johnson et al.,

1994) and; ii) synthesis or activation of specific transporters, possibly anion channels, to allow the organic-acid anions to be released from root cells (Delhaize, 1995).

Acid phosphatases are enzymes that cleave off esterified phosphate groups from organic substrates. They are normally localised in the cytosol, vacuoles or cell wall. Under P-deficiency stress, the activity of these enzymes in many plant species is increased and in some species, the enzymes are excreted (Goldstein et al., 1988; Duff et al., 1991). However, the exact role of extracellular phosphatase remains unclear because there is still doubt whether plants can utilise the major form of the organic phosphate (inositol hexaphosphate) in soil directly. Inositol hexaphosphate was reported to be a poor substrate for many characterised acid phosphatases from plants (Barret-Lennard et al., 1993; Richardson, 1994). Studies on *Brassica nigra* cell cultures under P deficiency showed that the increase in acid phosphatase activity was due to enhanced synthesis of the enzyme protein (Duff et al., 1991). This implies that synthesis of the protein is under either transcriptional or translational control by the P status of the plants. However, the molecular events that lead from sensing P deficiency to enhanced protein synthesis are not known.

Another factor that influences the P utilisation by plants in rhizosphere is the presence of microoganisms, mainly mycorrhizae which form hyphae in plant roots. Plants with mycorrhizal associations usually possess enhanced uptake of nutrients and this is especially the case for P (Smith et al., 1992). This is likely to be due to the formation of network of mycorrhizal mycelia which effectively increases the soil volume accessible to plant roots (Kothari et al., 1991). The mechanisms of Pi transport from the fungus to the plant are unknown. The mycorrhizal colonisation to a large extend is determined by rhizosphere conditions, which in turn are influenced by root exudations. For example, P deficiency can increase exudation of reducing sugars and amino acids and therefore stimulate the infection and growth of mycorrhizae (Graham et al., 1981). Mycorrhizal fungi were also reported to acquire P from organic sources that are not available directly to plant (Jayachandran et al., 1992).

1.3. P in plants

Phosphorus in plants can be divided into two major categories: organic phosphate and Pi. The main organic phosphate fraction includes DNA, RNA, P in lipids and P esterified to various compounds. The amount of each fraction will depend on the nature of the tissue. In young leaves, the proportion of the different groups is (as values in µg Pi / g fw): Pi:RNA:DNA:P-lipids:ester-P=10:2:0.2:1.5:1 (Bieleski, 1973). Although P concentrations vary among plant species and organs, the P concentrations in leaves of many species that is considered adequate for maximum yield is in the range of 0.2%-0.6% (Reuter and Robinson, 1986).

Changes in P supply to plants have a greater effect on the concentration of Pi than on the organic P fraction of the tissues. Bieleski (1968) found that transferring plants to a P-deficient medium decreased the organic P content by only about 4-fold, but the inorganic Pi concentration decreased 40-fold. Therefore, in plant analysis, the Pi fraction is a more sensitive indicator of the P status of the plant than total P.

Typical P deficiency symptoms are dark green foliage and reddening or purpling of leaves and petioles due to the accumulation of anthocyanins. In contrast, plants suffering from P toxicity become chlorotic and develop necrotic regions from the tips of their leaves.

1.4. Uptake and transport of Pi in plant

Plant roots take up Pi from the soil solution, unload Pi to the xylem and then transport it to shoots. Phosphate can also be translocated between different parts of shoots as well as down to the root via phloem. A range of different cell types and transport processes are likely to be involved in these processes.

1.4.1. Pi uptake by roots

The Pi concentration in the cytosol is about 10 mM (Lee et al., 1990; Mimura, 1995). When Pi enters plants, it needs to overcome a 10^3- to 10^4-fold concentration gradient which is greater than that found for other mineral nutrients (Bieleski, 1973). In addition to this concentration gradient, there is also the negative membrane potential across the plasma

membrane to be overcome, indicating that Pi uptake across the plasma membrane is an active process (Bieleski, 1973; Lefebvre and Clarkson, 1984; Ullrich-Eberius et al., 1984). Since Pi uptake is dependent on the extracellular pH and acidifies the cytoplasm, and is associated with membrane depolarisation (Ullrich-Eberius and Novacky, 1990; Sakano et al., 1992; Dunlop and Gardner, 1993), a current model proposes that $H_2PO_4^-$ is co-transported with protons (Sakano, 1990; Mistrik and Ullrich, 1996). The stoichiometry of H^+ co-transported with Pi across the membrane is still in argument with values of two or four being most likely (Ullrich et al., 1984; Sakano, 1990). In a notable exception, Reid et al. (1997) identified a $nNa^+/H_2PO_4^-$ symport mechanism operating in *Chara corallina*. A similar $nNa^+/H_2PO_4^-$ symport in higher plants has not yet been found.

There are many studies aimed at understanding the kinetics of Pi uptake by higher plants. Typically, the uptake rate of Pi has been found to follow Michaelis-Menten kinetics;

$$V=V_{max}C/(Km+C)$$

where Vmax is the maximal rate of uptake, C the concentration of Pi in the medium and Km is the Michaelis-Menten constant. Km is equal to the Pi concentration that gives half the Vmax and is an indicator of the affinity of the transport system. A wide range of microorganisms, algal cells and higher plant species follow this type of kinetics for Pi uptake (Epstein, 1976; Pitman, 1976; Burns and Beever, 1977).

Most of the studies suggest the existence of both high-affinity and low-affinity Pi uptake systems in plants (Barber, 1972; Bieleski, 1973; Epstein, 1976; Ullrich-Eberius et al., 1984; Dunlop et al., 1997). The high-affinity system with a Km of about 1 to 20 μM in many species is induced by P deficiency. By contrast, low-affinity uptake system operates over an extended range of concentrations with a Km of about 50 to 1000 μM (Drew et al., 1984). There are also examples of a single high-affinity transport systems (Lee, 1982; Shimogawara and Usuda, 1995) or multiphasic kinetic systems for the uptake of Pi (Nissen, 1991). Since the Pi concentration in soil is generally below 10 μM, the high affinity uptake system is likely to be operating under these conditions.

Root hairs and epidermal cell layers are likely to be involved in taking up Pi from the

external solution (Bieleski, 1973). Phosphate then travels across the root through the cytoplasm of the cortical cells to the vascular bundle where it is loaded into xylem vessels. The cells likely to be involved in the loading process are the xylem parenchyma cells (Jarvis and House, 1970; Bieleski, 1973).

1.4.2. Transport in xylem and phloem

Knowledge of the long distance transport to shoots mainly comes from the experiments using ^{32}Pi. In xylem, most of P is transported as Pi (Bieleski, 1973). It moves readily from xylem to phloem where the transport may be against a concentration gradient. Little is known about the movement of Pi from the phloem to xylem. The meristematic and young tissues are sinks for P in the plant. As the plant ages or becomes P deficient, Pi will be remobilised to these tissues to maintain growth. Another portion of Pi moves down the phloem into roots. Phosphate uptake has been reported to be regulated by shoot demand through this Pi recycled in the phloem (Drew and Saker, 1984; Marschner and Cakmak, 1986). A good demonstration showing that Pi is mobile within plants is the split-root experiments where one root was supplied with ^{32}Pi and the rest were starved of Pi (Drew and Saker, 1984). Compared with the P-fed control root, the single P-supplied root showed much higher ^{32}Pi uptake rate and translocated more Pi to shoot. The Pi concentration in the Pi-starved roots was similar as in the P-fed roots, indicating that supplying Pi to part of the roots may sustain the life of the plant due to Pi movement to other parts of plants including roots.

1.5. Compartmentation of P

1.5.1. The vacuole is the main storage of cellular Pi

At the cellular level, investigations of Pi compartmentation have been undertaken using isolated organelles (Stitt et al., 1985; Mimura et al., 1990). A disadvantage of these methods is the possibility of cross-contamination by leakage of Pi from organelles. For this reason, it is difficult to obtain a reliable estimation of Pi concentrations in cells. Experiments

using ^{32}Pi show that the cytoplasm and vacuole are the main compartments for Pi. The application of ^{31}P-NMR (nuclear magnetic resonance) has contributed most to determine the Pi compartmentation in cells. It allows direct detection of the cytoplasmic and vacular pools of Pi in living tissues due to the different pH environment of the Pi molecules. The typical pH of vacuoles is about 5.5 and the pH of cytoplasm is about 7 which produces a spectral shift for ^{31}Pi. The area under the NMR peak is proportional to the amount of ^{31}P in a particular environment allowing its quantitation. The vacuole has been identified as a main store of Pi (Ratcliffe, 1994). When deficient in P, cells usually maintain the concentration of cytoplasmic Pi at the expense of Pi in vacuoles (Rebeille et al., 1982; Lee and Ratcliffe, 1983; Lee et al., 1990). In yeast vacuoles, polyphosphate is the main form of P storage. Yeast cells that are starved of Pi continue to grow for 2-3 generations, while a mutant which is unable to accumulate Pi in the vacuole and therefore lacks polyphosphate stores, ceases growth immediately when exposed to P starvation (Shirahama et al., 1996). This work strongly confirms that the vacuole plays a critical role in maintaining Pi concentrations in the cell to allow metabolism to proceed.

1.5.2. Pi transport across the tonoplast

Phosphate transport from the cytoplasm into vacuoles is thought to occur by an energy-free process (Schachtman et al., 1998). This is based on the observation that Pi uptake in P-sufficient vacuoles was not greatly stimulated by ATP and no saturation of uptake was obtained in a range of Pi concentrations (Mimura et al., 1990). In addition, a H^{+}-translocating ATPase and a pyrophosphatase are known to pump protons into the vacuoles, generating a proton gradient and an electrical potential across the tonoplast that favours anion uptake into the vacuole (Sze, 1985). The Pi transport system in the tonoplast has not yet been characterised but it is possible that an anion channel permeable to Pi could be involved in Pi influx. In contrast to P-sufficient plants, where Pi uptake into vacuoles need not be energised, Pi uptake by P-deficient vacuoles was found to be ATP-dependent suggesting that different transport systems may operate in the tonoplast under different

conditions (Mimura et al., 1990). To date, there are no reports describing the kinetics of this transport system.

As noted before, cytoplasmic Pi is maintained nearly constant under P deficiency by exporting Pi from the vacuoles into the cytoplasm (Lee et al., 1990; Mimura et al., 1990). This process is against electrical potential and might be against the Pi concentration gradient as well. Little is known about Pi efflux from vacuoles but it is likely to require energy. Because of its important role in cells, Pi in the cytoplasm requires strictly controlled import of Pi to and export of Pi from the vacuole. The mechanisms that regulate the distribution of Pi between the cytoplasm and vacuole remain unsolved.

Because Pi concentration gradients and membrane potentials will differ across the membranes of various cells and cellular compartments, Pi transport across these membranes is likely to involve both energy-dependent and passive processes. Therefore, a range of Pi transporters including energy-dependent transporters and anion channels which allow the flow of Pi down its electrochemical gradient, are likely to operate in plants. Figure 1.1 shows Pi movement in leaf cells through the possible Pi transporters.
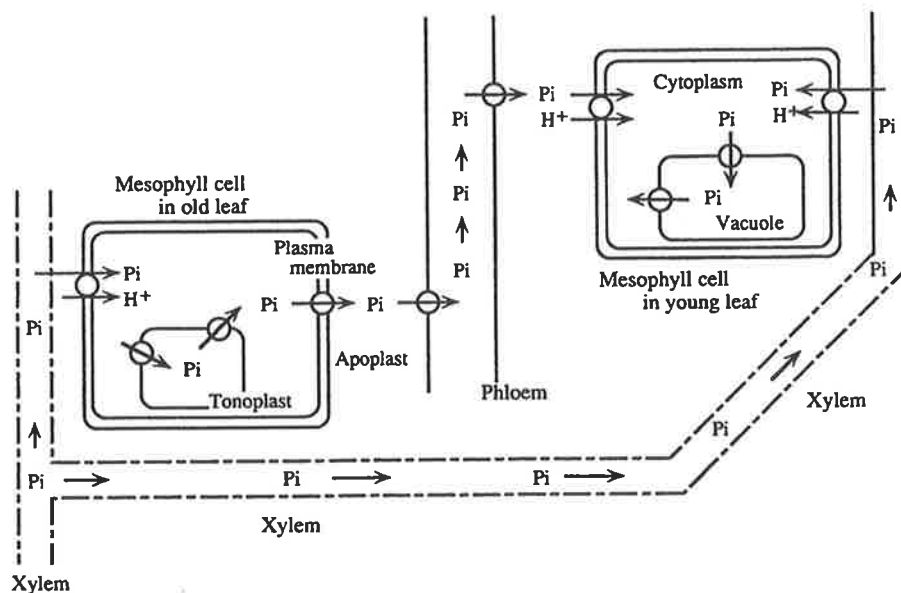
Fig. 1.1. Schematic diagram of Pi translocation and transport across membranes in a plant system (Mimura, 1995). Circles with two arrows denote the Pi transporters that are coupled to proton cotransport. Others denote possible transporters or channels in the plasma membrane and the tonoplast.

1.6. Regulation of P nutrition

Knowledge of the regulation of P nutrition in microbes at the molecular level may benefit similar studies in plants. Much is known of the molecular mechanisms involved in the regulation of P in microorganisms. In the prokaryote *Escherichia coli*, a *PHO* regulon system consisting of at least 25 genes is co-ordinately activated at the transcriptional level to cope with conditions of low phosphate in the medium (Torriani-Gorini, 1987). The expression of these genes is highly inducible by Pi limitation. These genes encode phosphatases, components of a high-affinity Pi transporter and other proteins involved in the transport and assimilation of Pi.

Regulation of Pi transport in yeast is also well-characterised and a similar *PHO* system has provided a basis for investigating signal transduction and transcriptional regulation in relation to P-nutrition. Since *Saccharomyces cerevisiae* is a eukaryote, it may serve as a model for plant studies.

1.6.1. Regulation of P in yeast

Although it is a single-celled organism, yeast has a complex system for P regulation. The system consists of products of a group of genes that include *PHO4, PHO80, PHO81* and *PHO85* which are regulated by Pi (Fig. 1.2). With Pi in the medium, Pho80p*, which is a cyclin-like molecule, forms a complex with protein kinase Pho85p. The Pho80p-Pho85p complex acts as a negative effector to aggregate with Pho4p through phosphorylation (Kaffman et al., 1994; Lenburg and O'shea, 1996). The phosphorylated form of Pho4p is unable to activate the transcription of *PHO8* and *PHO5* which encode an alkaline phosphatase (E.C.3.1.3.1.) and a repressible acid phosphatase (E.C.3.1.3.2.). Under P deficiency, however, Pho81p binds regions of DNA upstream of *PHO80* and *PHO85* and dephosphorylates the transcription activator Pho4p. In this case, Pho4p with a DNA binding protein Pho2p, binds to the promoter region of the structural genes *PHO5* and the repressible Pi transporter gene *PHO84*. The binding results in the loss of nucleosome

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* Nomenclature in yeast: *PHOX*--gene; Phoxp--protein.

structure in the promoter, which appears to be required to activate the promoter (Straka and

Hörz, 1991; Workman and Buchman 1993). Likewise, Pho4p and Pho9p activate *PHO8*

which encodes an alkaline phosphatase (Fig. 1.2). In this system, *PHO4* is an important

step in the regulatory mechanism because mutations in *PHO4* affect the expression of the

other *PHO* genes (Johnston and Carlson, 1991).
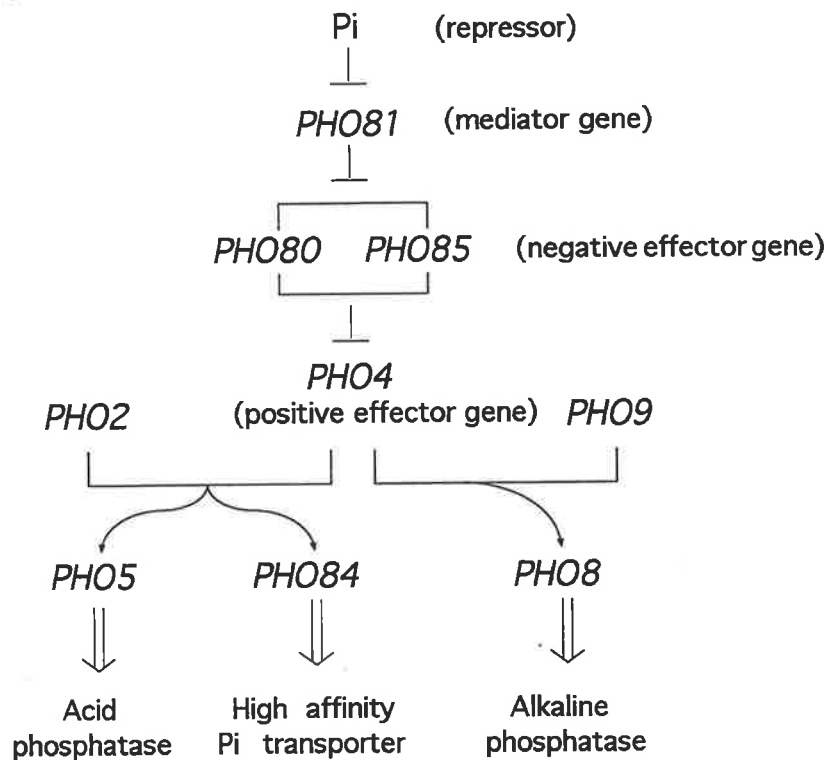
Fig. 1.2. Summary of the main elements involved in the Pi-responsive regulation in yeast. The bars denote inhibition of the genes and the arrows denote activation of the genes. The double arrows indicate genes encoding the corresponding proteins. The figure is based on those of Torriani-Gorini et al. (1987) and Johnson and Carlson (1991).

Similar to plants, there are two H^+/Pi transport mechanisms in *S. cerevisiae*; a high-affinity

system with a Km of 8.7 μM and a low-affinity system with a Km of 770 μM (Tamai et al.,

1985). *PHO84* encodes the high-affinity Pi transporter, and translation of its sequence

predicts a protein of 596 amino acids which shows homology to glucose transporters and

various other sugar transporters in mammals and bacteria (Bun-ya et al., 1991). Pho84p

consists of 12 transmembrane domains and has been shown to transport Pi in a pH-

dependent manner suggesting a H^+/Pi cotransport system (Bun-ya et al., 1991; Berhe et al., 1995). Antibodies raised against synthetic peptides corresponding to the N-terminal and the C-terminal regions of Pho84p displayed cross-reactions with the isolated plasma membrane vesicles of yeast (Fristedt et al., 1996). This has confirmed that the Pho84p is located in the plasma membrane which is consistent with its expected function. Pho84p has been reported to interact with a previously identified GTP-binding protein (Bun-ya et al., 1992) and other membrane proteins Pho86p, Pho87p and Pho88p (Bun-ya et al., 1996; Yompakdee et al., 1996) which are also transcriptionally regulated by the *PHO* system. The possible function of these membrane protein units is to sense phosphate starvation and to directly regulate the signal transduction pathway since Pho84p can bind DNA upstream of the mediator gene *PHO81* (Lenburg and O'shea, 1996).

In addition to the H^+-coupled phosphate transporter system, a high-affinity Na^+-coupled phosphate transport system has also been characterised (Rooman et al., 1977). Recent sequencing data from yeast chromosome 2 revealed a strong homologous sequence with several identified mammalian Na^+-phosphate symporters (Werner et al., 1991; Kavanaugh et al., 1994). This gene, designated *PHO89*, has been demonstrated to encode a derepressible high-affinity Na^+/Pi cotransporter (with a Km of 0.5 μM) that is also regulated by the *PHO* pathway (Martinez and Persson, 1998).

1.6.2. Regulation of P in other fungi

Since the isolation of the high-affinity phosphate transporter gene *PHO84* from yeast using the *pho84* mutant, homologous genes have been identified in other fungi. *GvPT* isolated from mycorrhizal fungus *Glomus versiforme* encodes a high-affinity phosphate transporter (Harrison and van Buuren, 1995). The function of the protein encoded by *GvPT* was confirmed by complementation of the yeast phosphate transporter mutant, *pho84*. The encoded protein of 521 amino acids contains 12 membrane-spanning domain, which is a typical structure for the family of solute transporters. The protein is not expressed in fungal structures inside plants, suggesting it is not responsible for Pi transport from the fungus to

the plant but is likely to be involved in uptake of Pi from the external medium (Harrison and van Buuren, 1995).

In *Neurospora crassa*, Pi limitation activates the synthesis of several proteins including acid phosphatase, encoded by *pho-3*[+*] , alkaline phosphatase, encoded by *pho-2*[+], and high-affinity Pi transporters encoded by *pho-4*[+] and *pho-5*[+]. *pho-4*[+] is different from other H^+/Pi transporter genes and shares little sequence identity with *PHO84, pho-5*[+] or *GvPT*. The hydropathy profile of the amino acids sequence of *pho-4*[+] suggests ten to twelve membrane-spanning domain helices (Mann et al., 1989). PHO-4 homologs were found in mammalian cells where they were shown to be phosphate transport proteins driven by a sodium symport (Kavanaugh et al., 1994). PHO-4 also shares strong sequence homology with the Na^+-coupled phosphate transporter PHO89 recently identified from yeast (Martinez and Persson, 1998). It is able to function to some degree without sodium ions, presumably using protons as a substitute (Versaw and Metzenberg, 1995) . PHO-5 shares 48% identity with the high-affinity phosphate transporter PHO84 in yeast (Versaw, 1995). The transcription of all these phosphate acquisition genes requires *nuc-1*[+], which is analogous to *PHO4* in yeast. The C-terminal portion of NUC-1 has been shown to bind specifically upstream of *pho-2*[+] and presumably does so to the upstream regions of the other *pho* genes as well (Peleg and Metzenberg, 1994). The *nuc-1*[+] knock-out mutant results in silencing of these genes indicating its critical role in controllingthe expression of these genes. *nuc-1*[+] is regulated by two other genes, *preg*[+] and *pgov*[+] (Metzenberg and Chia, 1979), which encode cyclin and cyclin-dependent kinase, possibly through phosphorylation like the transcription of *PHO4* regulated by the cyclin-dependent Pho85p in yeast, although there is no direct evidence for protein phosphorylation yet (Metzenberg, 1998). *preg*[+] and *pgov*[+] are regulated by another gene *nuc-2*[+], which is analogous to *PHO81* in yeast (Fig. 1.3).

Although the Pi-repressible signal transduction pathways have been well characterised in yeast and *Neurospora*, the common fundamental question on where Pi adequacy is initially sensed, i.e., where it interacts with PHO81 or NUC-2, is still not clear. Determination of

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* Nomenclature in *N. crassa*: *pho-x*--wild-type gene, PHO-X--protein

the cellular locations of these proteins with appropriate antibodies will provide information regarding their roles in Pi-regulation mechanism in these organisms.

Pi
⊥
nuc-2 [+]
⊥

pgov[+]   *preg*[+]

⊥
nuc-1 [+]

pho-2 [+]   *pho-3* [+]   *pho-4* [+]   *pho-5* [+]

| Alkaline phosphatase | Acid phosphatase | Na^+/Pi transporter | High-affinity Pi transporter |
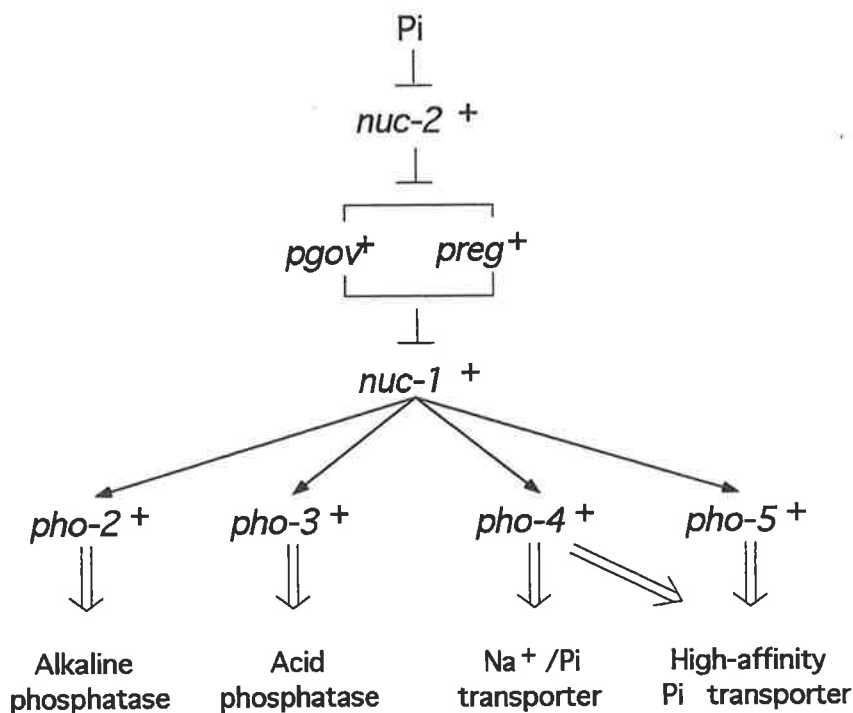
Fig. 1.3. Summary of the main elements involved in the Pi-responsive regulation in *Neurospora crassa*. The bars denote inhibition of the genes, and the arrows denote activation of the genes. The double arrows indicate the proteins encoded by the corresponding genes. The figure is based on Metzenberg and Chia (1979) and Metzenberg (1998).

1.6.3. Regulation of Pi homeostasis in plants

Plants respond to Pi starvation by increasing their Pi uptake capacity and the activities of several enzymes including acid phosphatase (Goldstein et al., 1989; Duff et al., 1991) and ribonucleases (Nurnberger et al., 1990; Löffler et al., 1992). Activities of other enzymes involved in photosynthetic carbon metabolism (Usuda and Shimogawara, 1992) and respiratory metabolism (Nagano and Ashihara, 1993; Theodorou and Plaxton, 1993) were also reported to be increased or repressed by P nutrition. Knowledge of the molecular basis responsible for Pi uptake and regulation is still limited in plants, although many studies

show that Pi uptake is related to internal Pi concentrations (Drew and Saker, 1984; McPharlin and Bieleski, 1989; Clarkson and Lüttge, 1991). Since Pi concentration in the cytoplasm does not decrease greatly during P deficiency, it is possible that either the decline in cytoplasmic Pi concentration is not responsible for activation of the various physiological processes or that small changes in cytoplasmic Pi concentration result in an increased uptake capacity. Vacuolar Pi may be involved in the signal transduction pathway because it is sensitive to changes in P-nutrition. However, it is difficult to explain how it could trigger the expression of genes encoding Pi transporters that are located in the plasma membrane. Alternatively, regulation of Pi uptake by external Pi concentration has also been proposed. Mimura et al. (1998) observed increased Pi uptake despite a relatively constant internal Pi concentration in *Chara corallina* cells, indicating that internal Pi, either cytoplasmic or vacuolar, is not likely to be responsible for regulating Pi uptake activity directly. In this case, one would suppose that a mechanism to sense Pi is located at the plasma membrane allowing the plant cell to sense the external Pi and to respond accordingly before the intracellular Pi concentration begins to drop. Obviously, more molecular evidence is needed to elucidate the mechanisms responsible for regulating Pi uptake in plants.

1.7. *Arabidopsis* research

Arabidopsis thaliana is a small cruciferous plant. Despite being of no economic value, it is widely used as a model plant in genetic research because of its short-life cycle (about 8 weeks), autogamy, large quantity of seeds and the most attractive feature, simple organisation of its genome which is well characterised. The diploid *Arabidopsis* nucleus contains five pairs of chromosomes. The haploid genome contains an estimated 7×10^7 base pairs of chromosomal DNA, which is about five times the size of the genome of the yeast *Saccharomyces cerevisiae* and 15 times that of the bacterium *Escherichia coli* (Meyerowitz, 1987). This is small compared with genomes of other higher plants such as tobacco and pea whose haploid genome sizes are 1.6×10^9 bp and 4.5×10^9 bp, respectively (Wilson et al., 1991). The simple organisation of the genome has important implications for cloning genes from *Arabidopsis* by procedures such as chromosome walking and transposon tagging.

Arabidopsis is one of a few plant species where it is possible to clone a gene based solely on a mutant phenotype. Furthermore, *Arabidopsis* genome sequencing is underway and is expected to be completed by the year 2004 or earlier (Website: http://genome-www.stanford.edu/ Arabidopsis/AGI/AGI_memo.html). This will greatly accelerate the cloning of genes responsible for a range of processes in *Arabidopsis* and will allow the cloning of homologous genes from other higher plants.

In recent years, *Arabidopsis* has been used to study aspects of mineral nutrition. A number of mutants have been identified including mutants in nitrate uptake (Tsay et al., 1993), Cd sensitivity (Howden and Cobbett, 1992), Mn accumulation (Delhaize, 1996), Cu sensitivity (van Vliet et al., 1995) and P nutrition (Poirier et al., 1991; Delhaize and Randall, 1995).

1.7.1. The *pho1** mutant

The first P-nutrition mutant of *Arabidopsis* was isolated by Poirier et al. (1993). The recessive mutant, named *pho1*, accumulates about 5% of the Pi and 24-44% of the total P as wild-type plants (Poirier et al., 1991). The Pi concentration in roots of *pho1* is similar to that in wild-type plants. The growth of the mutant is reduced compared to wild-type plants and it exhibits typical P deficiency symptoms. The uptake rate of Pi by the intact mutant was similar to that of wild type but the amount of Pi transferred to shoot was only 3 to 10% of the wild-type levels when the external Pi concentration was 200 µM. There was no significant difference in Pi uptake into hypocotyls of *pho1* and wild type. From these investigations, Poirier et al. (1993) suggested that *pho1* is impaired in xylem loading of Pi. The exact role of *PHO1* in Pi nutrition would be clearer if the gene is cloned and its product characterised. Another mutant, which is an allele of *pho1*, was designated as *pho1-2* and was mapped to chromosome 3 (Delhaize and Randall, 1995).

1.7.2. The *pho2* mutant

The recessive *pho2* mutant accumulates up to 2- to 5-fold more total P in leaves, mostly as

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* Nomenclature in *Arabidopsis*: *phox*--mutant gene, *PHOx*--wild-type gene, and PHOx--protein.

Pi, than wild-type plants. The Pi concentrations in stems, siliques and seeds of *pho2* are also higher while the Pi concentration in roots is similar to wild-type plants (Delhaize and Randall, 1995). Under high transpiration conditions, *pho2* mutants show P toxicity symptoms suggesting an inability of these plants to regulate leaf Pi concentrations. The *pho2* locus was mapped to chromosome 2 by using both phenotypic and cleaved amplified polymorphic sequence (CAPS) markers for the various chromosomes (Delhaize and Randall, 1995). Double mutants resulting from a cross between *pho1* and *pho2* show the *pho1* phenotype. This result is consistent with the hypothesis that *pho1* mutant is deficient in loading Pi into xylem and that *pho2* is specifically involved in regulating leaf Pi concentrations. The physiological defect responsible for *pho2* phenotype has not yet been identified.

1.8. Aims of the project

A detailed study of the *pho2* mutant and other P nutrition mutants of *Arabidopsis* will provide information on the function of genes involved in P nutrition and their regulation in higher plants. In this project, investigations of Pi uptake, transport and other P-related physiological processes in *pho2* mutants were conducted by comparison with wild-type plants. The specific aims of the project are:

1) to further define the defect in *pho2* mutants that results in accumulation of Pi in leaves;

2) to characterise Pi transporter genes isolated from *Arabidopsis* and to study their relationship with *pho1* and *pho2*; and

3) to fine-map *PHO2* on chromosome 2 as a precursor for cloning the gene.

Chapter 2

Possible processes related to Pi accumulation in pho2 mutant

2.1. Introduction

Plants usually regulate their Pi concentrations in leaves within a limited range regardless of external Pi concentrations. For example, wild-type *Arabidopsis* had similar Pi concentrations in their leaves when grown with a range of Pi supplies (5 to 250 µM). However, under the same conditions, the *pho2* mutant accumulates excessive Pi in its shoots (Delhaize and Randall, 1995). By contrast, there were no differences in Pi concentrations in roots between wild type and the mutant. In this chapter, several factors that could be related to Pi accumulation in *pho2* mutants were assessed. These factors included Zn deficiency and increased acid phosphatase activity. In addition, the location of excess Pi in shoot cells of the *pho2* mutant was also examined.

Zinc deficiency has been reported to enhance Pi uptake by roots and its subsequent translocation to shoots (Christensen and Jackson, 1981; Loneragan et al., 1982; Marschner and Cakmak, 1986, Loneragan and Webb, 1993). This phenomenon is specific for Zn deficiency and does not occur in plants deficient in other micronutrients such as Fe, Cu, or Mn. These results indicate that Zn is important in the control of Pi uptake and translocation. The mechanisms for enhanced Pi uptake in Zn-deficient plants are still obscure. It has been proposed that Zn is necessary for the integrity of the root cell membrane and that under Zn deficiency the membrane becomes more permeable resulting in excessive Pi uptake by roots (Safaya, 1976; Welch et al., 1982). However, it is unlikely that a damaged membrane would affect only P influx. Also, it is known that the Pi concentration within the cell is much higher than in the environment (Mimura et al., 1990), and that Pi uptake across the plasma membrane is against a concentration gradient. Therefore, if membrane integrity is affected by Zn deficiency, an efflux of Pi out of root cells would be expected. Another explanation for P accumulation induced by Zn deficiency comes from the investigations

conducted by Marschner and Cakmak (1986). They observed that in Zn-deficient plants, Pi retranslocation from the shoot to the root, which may indirectly control Pi uptake by roots, was impaired. They hypothesised that Zn plays a critical role in providing the signal to recycle Pi in the phloem. The excessive accumulation of Pi to toxicity levels in the case of *pho2* mutants shows similarities to the P toxicity induced by Zn deficiency. Therefore, it is possible that the *pho2* phenotype is a result of Zn deficiency. Zinc concentrations in shoots of *pho2* plants have been analysed at a single growth stage and were found to be similar to wild type (Delhaize and Randall, 1995). However, no comparable data for roots was reported. In this chapter, Zn concentrations in both shoots and roots of *pho2* mutant and wild-type plants were monitored over a range of growth stages to investigate in detail whether Zn deficiency could account for P accumulation in the *pho2* mutant.

The accumulated Pi in *pho2* plants is located in leaves. At the cellular level, the location of this excess Pi has yet to be identified. The Pi might be found in the apoplast outside the cells or taken up into cells which will lead to different explanations for the *pho2* phenotype. In addition to the studies on Zn in relation to *pho2* mutants in this chapter, the location of the excessive Pi in *pho2* leaves was investigated by analysing Pi concentrations in protoplasts from *pho2* and wild-type plants. Another phosphate mutant, *pho1*, which is deficient in Pi (Poirier et al., 1991) was also used in this study. The *pho1* has less than 5% of Pi in its leaves compared with wild type, while the Pi concentration in roots in the two genotypes are similar. It was suggested that *pho1* is defective in xylem loading of Pi. If this is the case, the Pi concentrations in *pho1* leaf cells should be low, and, if they are supplied with external phosphate, the Pi concentration in *pho1* protoplasts should increase.

Phosphate deficiency has been reported to increase the activity of several enzymes including acid phosphatases (Goldstein et al., 1988; Duff et al., 1991) and ribonucleases (Taylor et al., 1993). These enzymes are proposed to be the components of the "Pi rescue system" and are suggested to enhance the remobilisation of Pi within plants under P deficiency. It is possible that the *pho2* mutant is a "derepressed" mutant with genes encoding these enzymes turned on all the time regardless of Pi levels in the cells. In this chapter, the acid phosphatase activity from *pho1*, *pho2* and wild-type plants was examined by native

polyacrylamide gel electrophoresis (PAGE) to investigate whether there are differences between the genotypes.

2.2. Materials and methods

2.2.1. Plant growth

The *pho2* mutant (Columbia genetic background) was back-crossed at least three times to wild-type *Arabidopsis* before being used in the physiological experiments. For hydroponic culture, seeds were germinated on plugs of rockwool that were dipped into nutrient solution as described by Delhaize and Randall (1995) (Fig. 2.1). For each plug of rockwool, nine seeds were sown and thinned to four plants one week after germination. The nutrient solution contained (in μM): 625 KNO_3, 250 $CaCl_2$, 250 $MgSO_4$, 250 KH_2PO_4, 6.25 Na_2EDTA, 6.25 $FeCl_3$, 11.5 H_3BO_4, 2.7 $MnCl_2$, 0.35 $ZnSO_4$, and 0.3 $CuCl_2$, at a final pH of 6.0. The nutrient solution was aerated and plants were grown under continuous light provided by fluorescent light tubes (photosynthetically-active radiation at plant top was 100 - 150 μmol photons m^{-2} s^{-1}) at about 20 °C. The nutrient solution was renewed two weeks after sowing and then every 3 d as well as on the day before the experiments.

For agar-grown plants, seeds were sterilised in a solution containing 5% (v/v) H_2O_2 and 50% (v/v) ethanol for 20 min and then rinsed thoroughly with sterile water. Twenty seeds were then sown on 100 ml autoclaved 0.75% (w/v) agar (Bacto-Agar; Difco Laboratories, Detroit, MI) in a 14-cm diameter glass petri dish. The agar contained 29 mM sucrose and mineral nutrients at concentration 4-fold of that used in hydroponic culture with the pH adjusted to 6.0. The growth conditions were the same as for the hydroponic cultures described above.

2.2.2. Phosphate and Zn assays

Plants grown in solution culture were harvested at 22, 36, 45 and 53 d after sowing. The bulked leaves and roots (washed in 0.2 mM $CaCl_2$) were collected separately in preweighed acid-washed tubes and dried at 70 °C overnight. After weighing, plant material was ashed at

540 °C overnight in a furnace. Concentrated nitric acid and 30% H_2O_2 (100 µL of each) were then added to the ash. The mixture was further digested on a hot plate at 90 °C for several hours until near dryness. The volume was then made up to 5 ml (or 3 mL depending on the amount of plant material) with distilled water for total P and Zn assays. Inorganic phosphate in fresh shoots and roots was extracted with 5 M H_2SO_4 (40 µL/20 mg fw). The mixture was diluted with distilled water to 3 ml and filtered through Whatman number 4 filter paper. Total P and Pi were analysed using a colourimetric assay with a malachite green reagent (Irving and Bouma, 1984). The absorbance was measured at 650 nm on a Varian Techtron UV-Vis spectrophotometer (model 635) at least 30 min after adding the reagent to samples. Total Zn was analysed by flame atomic absorption spectroscopy (Perkin-Elmer Cetus). Because there was insufficient material from the harvest of 22-d old plants, only Pi was assayed in these plants.

2.2.3. Enzyme purification

The commercial enzymes used for protoplast isolation (cellulase "Onozuka" R-10 and macerozyme R-10, Serva) contain high Pi concentrations and needed to be purified by gel filtration before being used. A gel column of Sephadex G-50 (3 cm diameter × 10 cm height) was equilibrated with 1 mM MES (pH 5.5). The concentrated enzyme solution containing 12% cellulase and 3% macerozyme was loaded on the column (about 4 mL). Two ml aliquots of effluent were collected in tubes and assayed for protein concentration by absorbance at 280 nm and by a coomassie blue method (Bradford, 1976). Inorganic phosphate was assayed using the malachite green reagent described above (Irving and Bouma, 1984). The tubes containing the protein peak were combined and the protein recovered was compared with the amount of protein in the sample originally loaded. The final enzyme solution was diluted so that it contained 1% cellulase and 0.25% macerozyme in 8 mM $CaCl_2$ and 0.4 M mannitol. Both purified and non-purified enzyme preparations were used to isolate protoplasts.

2.2.4. Protoplast isolation

Protoplasts were isolated from leaves of agar-grown plants according to the method described by Altmann et al. (1992) with slight modifications. Leaves of *Arabidopsis* were cut with a new razor blade into 3-4 pieces in 0.5 M mannitol. The mannitol solution was removed by pipette and the enzyme digestion solution was added to the leaf pieces (10 mL/0.5 g fw). The mixture was incubated in the dark at 25 °C overnight without shaking. The next day the mixture was gently shaken to release the protoplasts and incubated for a further 30 min. The mixture was then sieved through 125- and 63-μm metal meshes. The remaining leaf pieces were rinsed with 0.5 volume of 0.2 M $CaCl_2$, and the solution was also filtered through the metal mesh. The combined filtrates were centrifuged at $60 \times g$ for 5 min in a swinging-bucket rotor (Megafuge 1.0R, Heraeus Sepatech). The pellet was resuspended and washed with 0.5 M mannitol : 0.2 M $CaCl_2$ (v:v=2:1). The supernatant of each wash was assayed for Pi to monitor Pi released by broken protoplasts during the washes. The final protoplast pellet was resuspended in 0.5 M mannitol to a final density of about 10^5 cells/mL as determined by counting protoplasts with a haemocytometer. The protoplasts were kept on ice before being assayed for Pi, protein and chlorophyll content.

The protoplasts were lysed with distilled water, vortexed completely and centrifuged at $10,000 \times g$ for 10 min. The supernatant was assayed for Pi as described in the above section (2.2.2.) and protein was assayed according to the method described by Bradford (1976). Chlorophyll was extracted with 80% acetone and the content was calculated according to the equation described by Arnon (1949). Phosphate concentrations in protoplasts were then calculated on the basis of protein, chlorophyll or cell numbers.

2.2.5. Activity gel for acid phosphatase

Leaves of plants grown on agar were homogenised with 0.1 M, HEPES extraction buffer (pH 7.4 , 1 g plants/1 mL buffer) containing 0.5 mM DTT and 1 mM EDTA. The mixture was centrifuged for 15 min at $17,000 \times g$ at 4 °C and the resulting supernatant was used for electrophoresis.

Native PAGE was performed using 7.5 % (w/v) polyacrylamide slab gels (Gabard and

Jones, 1986). After electrophoresis, the gel was washed three times with 0.1 M sodium acetate (pH 5.2) at 4 °C over 10 min for each wash. The gel was incubated in 0.1 M sodium acetate (pH 5.2) buffer containing α-nathyl acid phosphatase (1 mg/mL) and Fast Blue RR (1 mg/mL, Sigma) at 37 °C for 20 min. The enzyme reaction was then stopped by replacing the substrate with 10 % (v/v) acetic acid.

2.3. Results and Discussion

2.3.1. Phosphate and Zn concentrations in intact plants

Growth of *pho2* mutant and wild-type was similar at early stages. About 30 d after sowing, P toxicity symptoms on the *pho2* mutants consisted of marginal necrosis on leaves (Fig. 2.2). The symptoms became more severe as *pho2* mutants aged and differences in growth between *pho2* and wild-type seedlings become apparent with *pho2* seedlings being smaller than wild type.

Total P concentrations in *pho2* shoots were 2- to 4-fold greater than in wild-type shoots over the course of the experiment (Fig. 2.3a). Similarly, shoot Pi concentrations were 3- to 6- fold greater in *pho2* mutant than in wild type (Fig. 2.3c). By contrast, total P and Pi concentrations (Fig. 2.3b, d) in roots of *pho2* mutants were similar to wild-type seedlings, confirming that *pho2* mutants specifically accumulate Pi in shoots (Delhaize and Randall, 1995).

At each harvest, Zn concentrations in shoots and roots of *pho2* plants were similar to those of wild-type plants (Fig. 2.4). The critical concentration of Zn required in shoots for maximum yield in many plant species is of the order of 20 μg/g dw (Reuter and Robinson, 1986). The 35-50 μg Zn/g dw recorded in the present experiment for both *pho2* and wild type shoots was well within this range. Phosphate toxicity symptoms in *pho2* mutants were severe under these conditions, but it was clear that the toxicity was not caused by Zn deficiency in either roots or shoots. However, it is still possible that internal use of Zn is defective in relation to Pi transport or translocation in the *pho2* mutant and this would not be apparent by measuring total Zn concentrations of tissues.
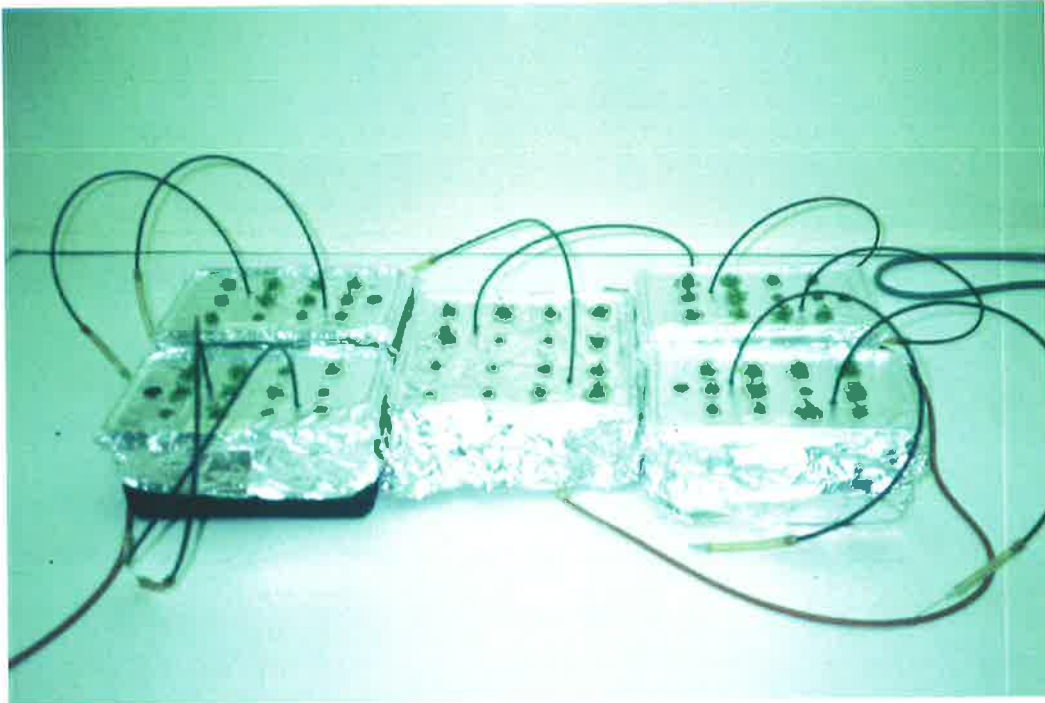
Fig. 2.1. *Arabidopsis* grown in hydroponic culture.

Fig. 2.2. Thirty-day-old *Arabidopsis* plants of wild type (left) and *pho2* mutants (right). The *pho2* mutants show necrosis along margins of leaves, typical symptoms of P-toxicity.
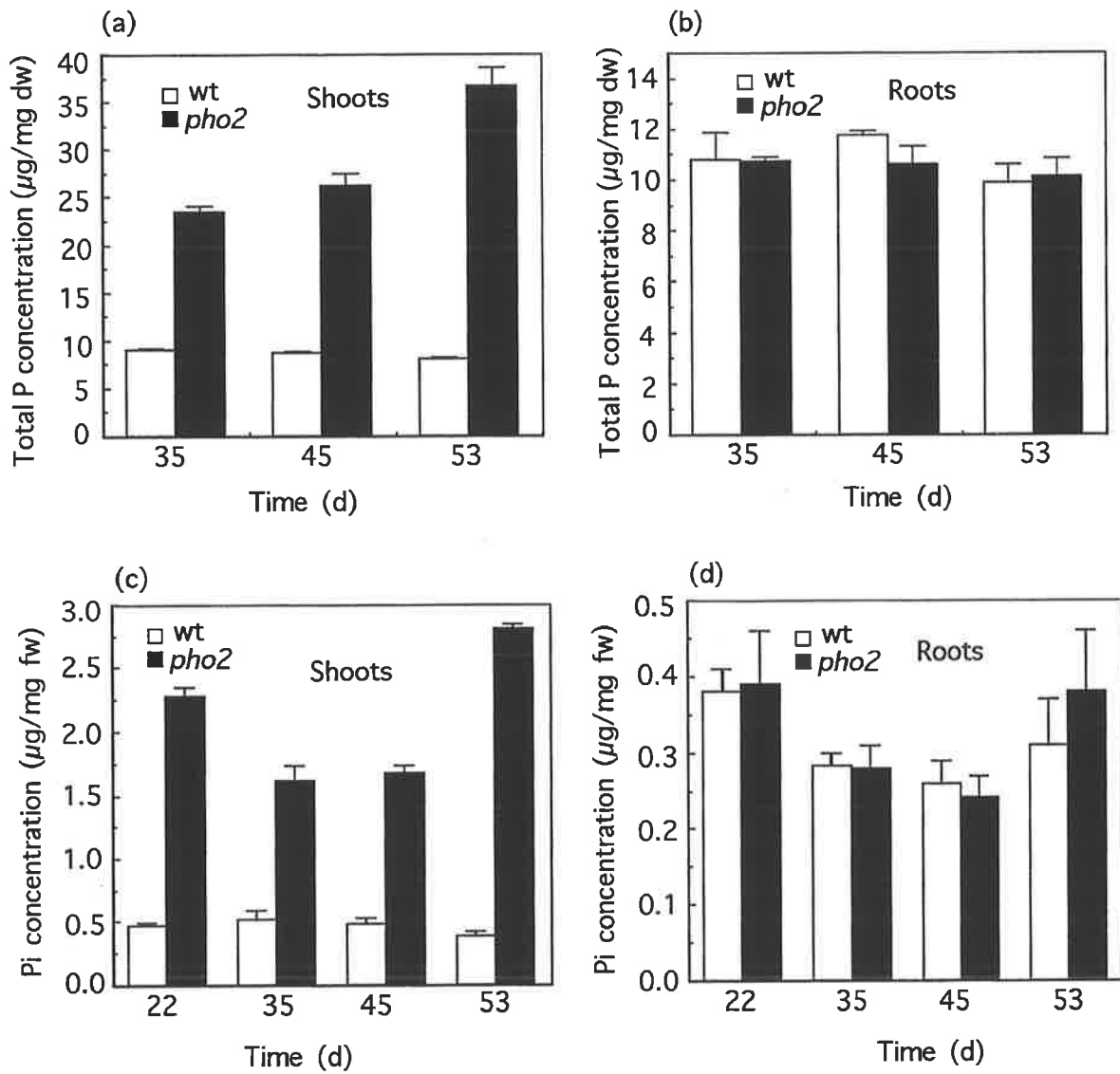
Fig. 2.3. Total P concentrations in shoots (a) and root (b); and Pi concentrations in shoots (c) and roots (d) of *pho2* and wild-type (wt) plants at different stages of growth. The error bars represent the standard errors of the means from 3 to 4 replicates.
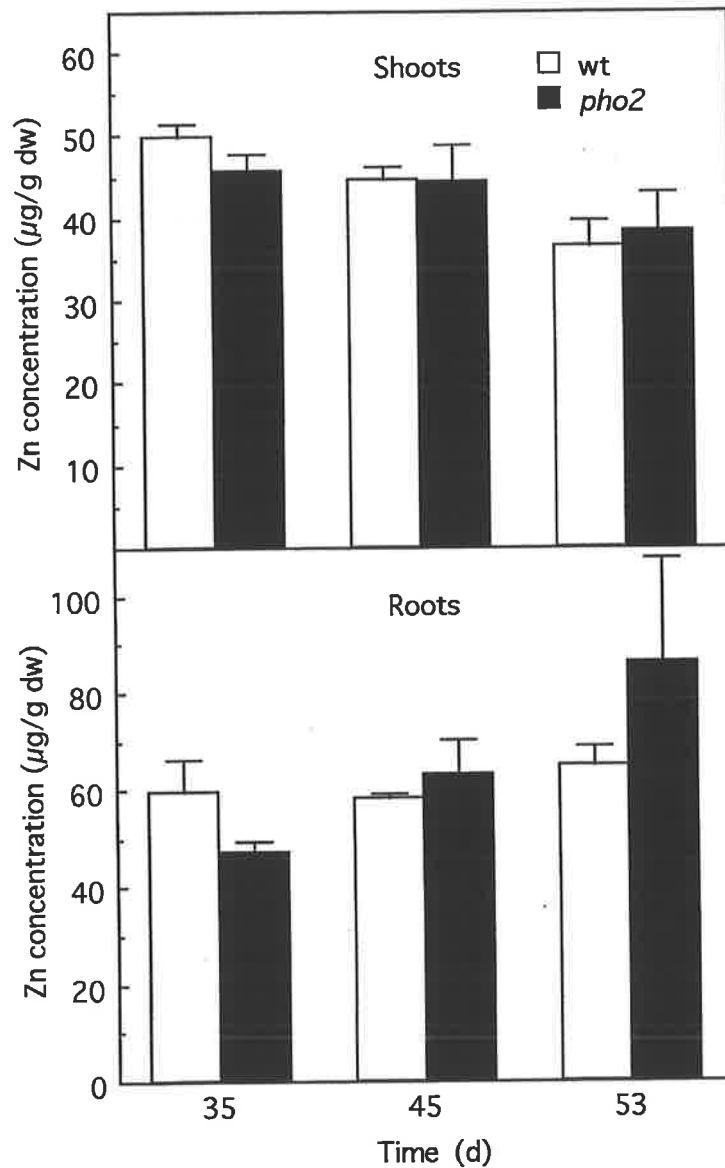
Fig. 2.4. Total Zn concentrations in *pho2* and wild-type plants (wt) at different stages of growth. The error bars represent the standard errors of the means from 3 replicates.

2.3.2. Phosphate concentration in protoplasts

Enzymes used for protoplast digestion were purified by Sephadex G-50 to remove contaminating Pi. Figure 2.5 shows two well-separated peaks that represent enzyme protein and Pi. Purified enzyme solutions were usually diluted about 4- to 6-fold.
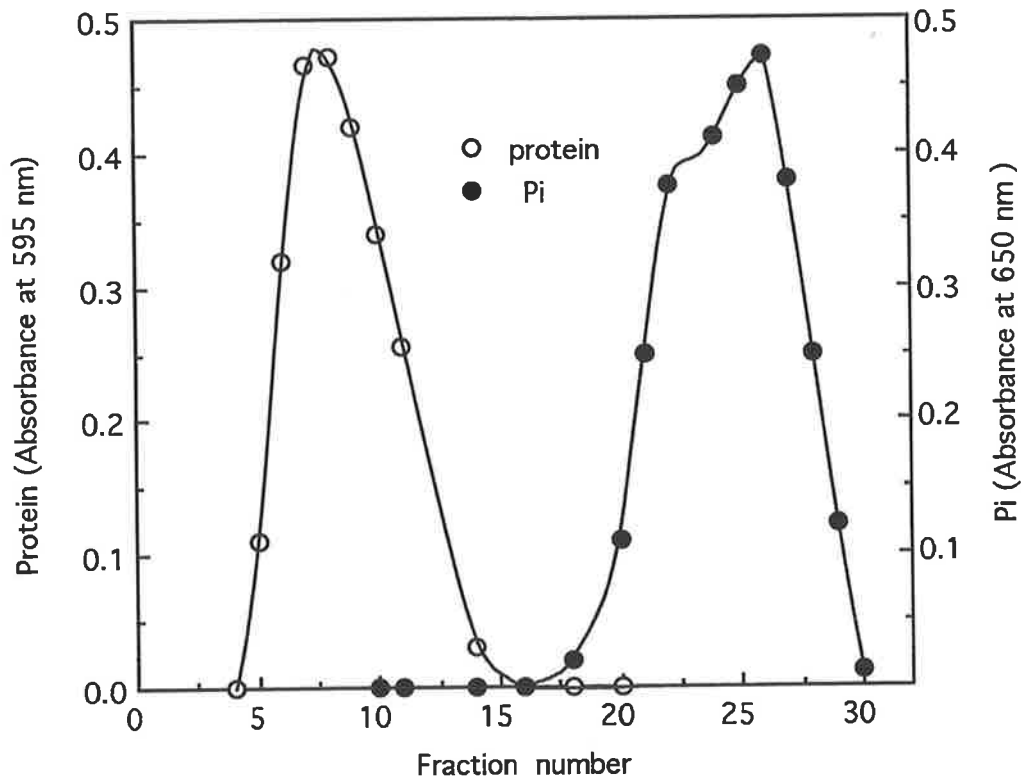
Fig. 2.5. Purification of enzymes used for protoplast isolation by Sephardex G-50. Protein was assayed by coomassie blue (Bradford 1976) and Pi was assayed by malachite green reageant (Irving 1984)

In a typical experiment, about 10^5 to 10^6 protoplasts were obtained from 0.2 - 0.4 g leaves (Fig. 2.6). There was no difference between the yields of protoplast isolated by purified and non-purified enzyme suggesting that the purification procedure did not reduce the activity of the enzymes.

Wild type, *pho1* and *pho2* plants grown on agar were used for isolation of protoplasts. The growth of *pho1* mutants on agar was reduced compared to the other genotypes due to P deficiency and they had very low leaf Pi concentrations of 0.03 µg/mg fw, which was about 4% of wild-type plants (Fig. 2.7). For *pho2* mutants, the Pi concentration in leaves was more than 2-fold greater than wild type (Fig. 2.7). This was consistent with the results obtained from the hydroponically-cultured plants (Fig. 2.3) although the differences between *pho2* mutants and wild type were not as great. The different Pi concentrations
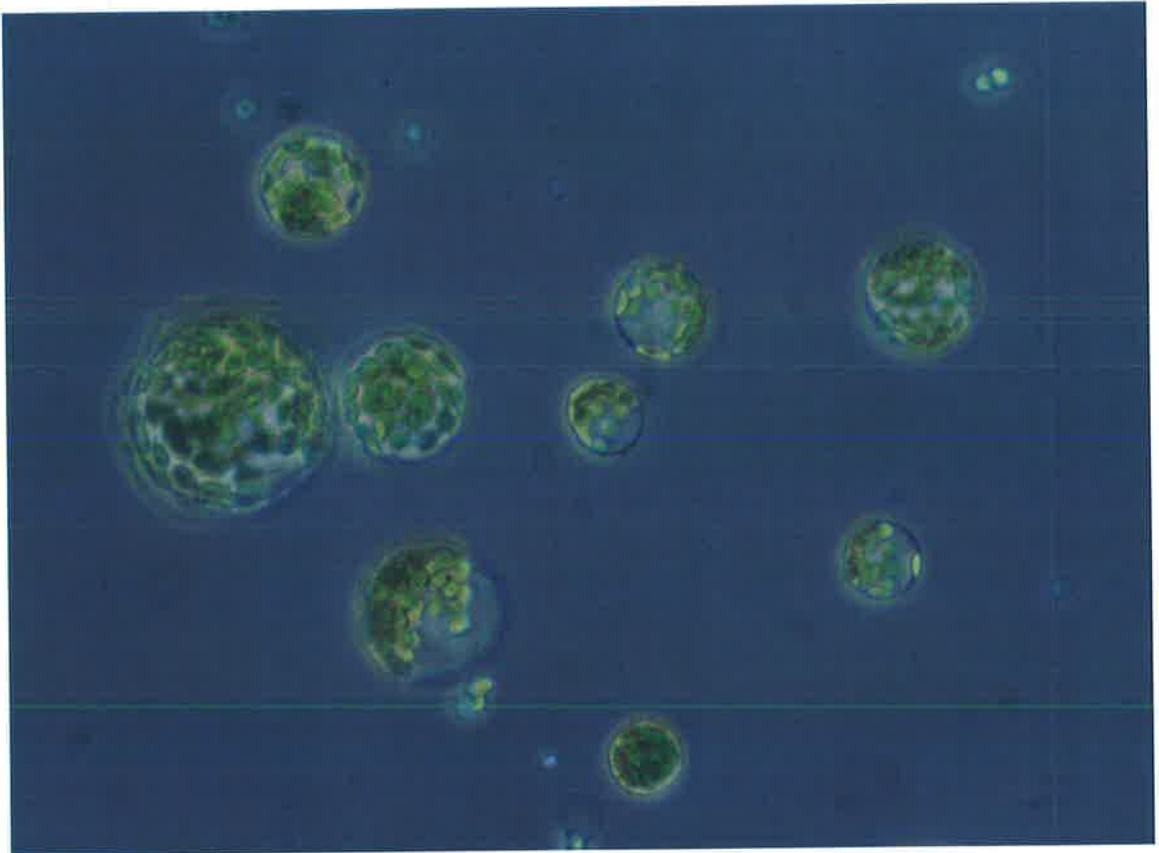
Fig. 2.6. Protoplasts isolated from leaves of wild-type *Arabidopsis* grown on agar medium. Photograph at 443X magnification.

accumulated in *pho2* mutants when grown in agar compared to hydroponic culture could be explained by different transpiration rates under the two conditions. Agar-grown plants were grown in a sealed environment where the plants had very low transpiration rates resulting in a lower accumulation of Pi. Regardless of the growth conditions, *pho2* mutants had considerably greater Pi concentrations in shoots than wild-type plants.
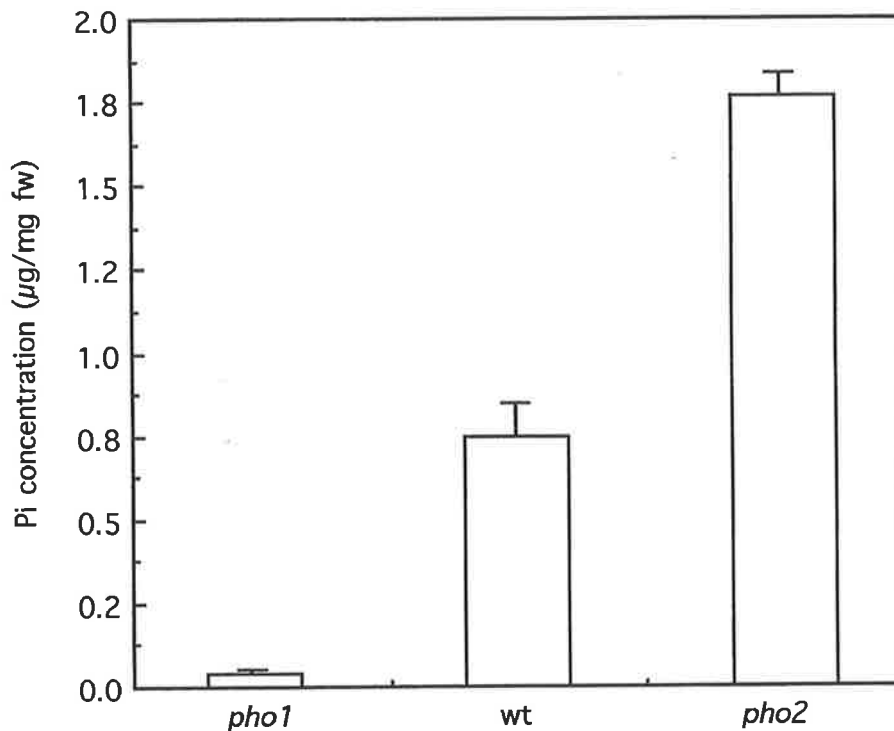
Fig. 2.7. Pi concentration in leaves of *pho1*, wild type (wt) and *pho2* plants grown on agar for 23 d. The error bars represent the standard errors of the means from 3 replicates.

During the process of isolating protoplasts, it was inevitable that some would burst and release Pi into the medium. To check if the Pi from the burst protoplasts might contribute the final Pi measurement of lysed protoplasts, supernatant solutions from each wash of protoplasts were assayed for Pi concentration. Table 2.1 shows that the Pi concentrations decreased with successive washings and remained at a low level after the third wash. Phosphate concentration in the third wash solution (0.078 µg/mL) was less than 2% of the total Pi concentration released from protoplasts lysed by distilled water (more than 5 µg/mL). Therefore, this part of Pi could be ignored when determining internal Pi

concentrations. Protoplasts were routinely washed three times before being lysed for the Pi

assay.

Table 2.1. Pi concentration in the wash solutions of protoplasts. Supernatant of
0.2 mL was assayed using a malachite green reagent (Irving and Bouma, 1984).

Wash number	1	2	3	4	5
Pi concentration (μg/mL)	0.425	0.175	0.078	0.083	0.075

Phosphate concentrations in protoplasts prepared from *pho2* mutants were consistently

greater than in those prepared from wild-type plants regardless of whether the

concentrations were expressed on the basis of chlorophyll, protein or cell number (Fig.

2.8). This reflected the differences between the genotypes in total Pi concentrations of their

Fig. 2.8. Pi concentrations in protoplasts isolated from wild type (wt) and *pho2*
plant leaves with purified enzymes. The error bars represent the SE of the means
from 3 replicates except for the data based on cell number which only had one
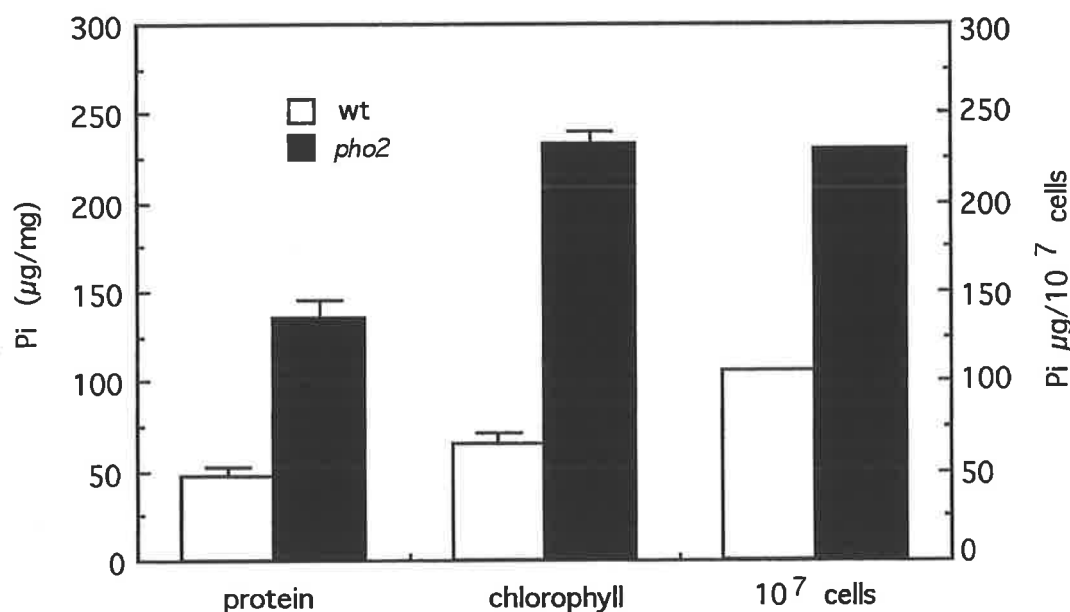replicate.

leaf tissue. The result indicates that the symplastic Pi in *pho2* leaves is greater than in wild type. The possibility that higher Pi concentrations also exist in the apoplast can not be excluded. Unfortunately, efforts to measure Pi in the apoplast of *Arabidopsis* using the method described by Mimura (1990) for barley were unsuccessful.

To further investigate the location of excess Pi in leaf protoplasts of *pho2*, I tried to isolate vacuoles from protoplasts to determine the vacuolar Pi concentrations compared to wild type. However, the number of vacuoles isolated by several methods described for other plant species were not be sufficient to obtain reliable results. ^{31}P-nuclear magnetic resonance (Lee et al., 1990) can be used to discriminate vacuolar Pi from cytosolic Pi as an alternative to isolating vacuoles, and measuring the Pi concentration directly. However, this technique was not available to my project. It is known that vacuoles are used to store Pi in cells and when plants are P-deficient, the cytoplasmic Pi concentration is kept constant by accessing Pi in the vacuoles. This indicates that a stable concentration of cytoplasmic Pi is necessary for maintaining normal cell metabolism. Although *pho2* mutants accumulate Pi in leaf cells and the plants show P toxicity symptoms under high transpiration conditions, they are still able to complete their life cycle and set seed which suggests that cell metabolism in *pho2* is not severely disturbed. Therefore, it is likely that the excess Pi in *pho2* leaf cells resides mainly in the vacuoles and not the cytoplasm.

Inorganic phosphate concentrations in *pho1* mutants were too low to be detected in the protoplasts isolated by the purified enzymes. Protoplasts isolated by non-purified enzyme were also used to measure Pi. These data showed detectable Pi concentrations in *pho1* leaf protoplasts (Fig. 2.9). The increased Pi concentration in *pho1* leaf protoplasts could be attributed to uptake of the Pi from the non-purified enzyme solution which contained a high Pi concentration, indicating that leaf protoplasts of *pho1* mutants were functional for Pi uptake. This result is consistent with the hypothesis that the *pho1* mutant is defective in Pi loading in xylem (Poirier et al., 1991), but is functional for Pi uptake by shoot cells.

2.3.3. Acid phosphatase activity

Acid phosphatase activity gels showed that the *pho1* mutant, which is P-deficient in shoots, had three isoenzymes present which are absent from wild type and *pho2* mutants (Fig. 2.10). Since *pho1* mutants are P-deficient in their leaves, the increased acid phosphatase activity in leaves of *pho1* mutants is consistent with the results obtained from other plant species under P deficiency (Goldstein et al., 1989; Duff et al., 1991). If the *pho2* mutant is a derepressed mutant overexpressing genes that encode acid phosphatase, then a similar
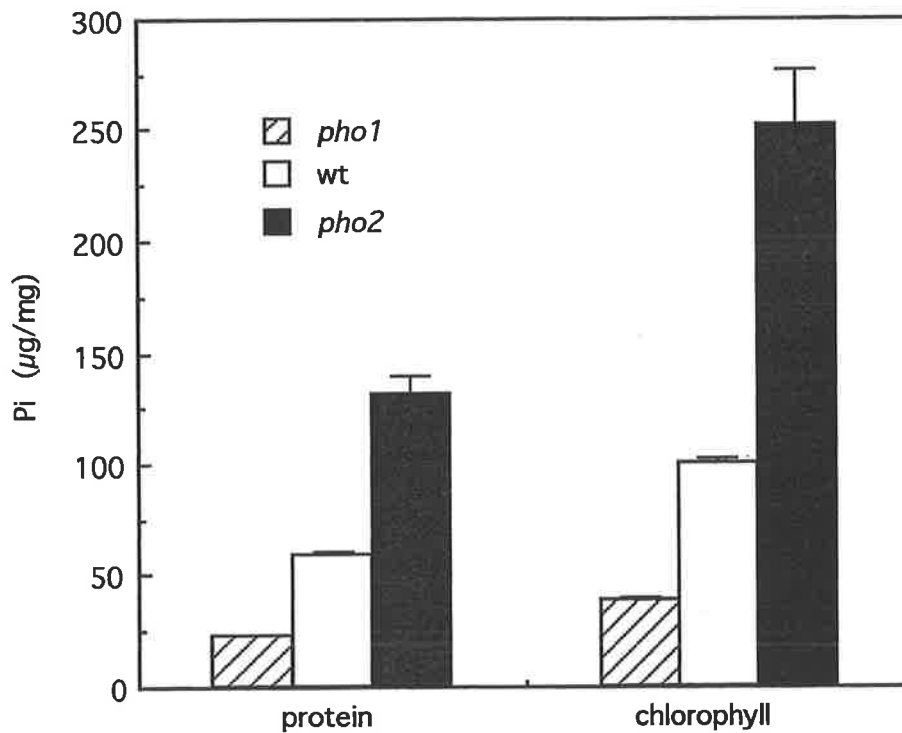
Fig. 2.9. Pi concentrations in protoplasts isolated from *pho1*, wild type (wt) and *pho2* leaves with non-purified enzymes. The error bars represent the standard errors of the means from 3 replicates.

increase in acid phosphatase activity as in *pho1* mutants would be observed. However, there were no differences in acid phosphatase activity in leaves of *pho2* mutants compared with wild-type plants, suggesting that *pho2* is not likely to be a general derepressed mutant. This finding is consistent with the result of McNight et al. that expression of an acid phosphatase gene in P-fed *pho2* leaves is similar to that in wild-type leaves (personal communication).
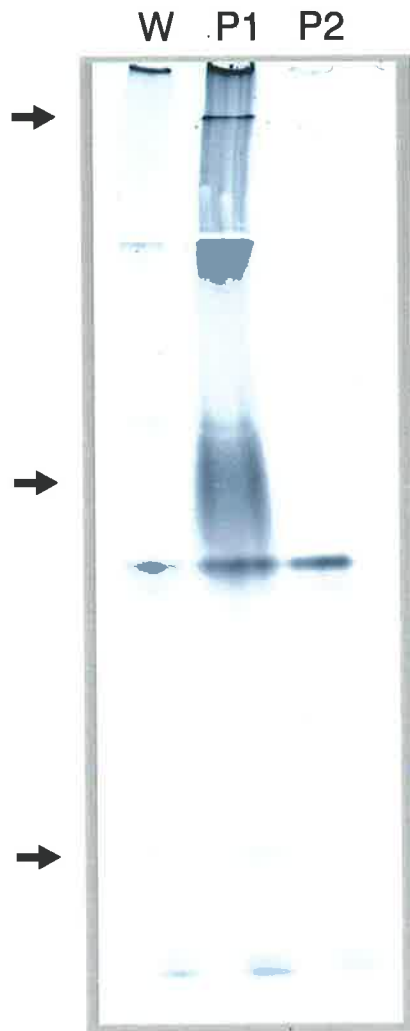
Fig. 2.10. Acid phosphatase activity stain of native PAGE gel (31.5 µg protein/per lane). Proteins were extracted from leaves of wild-type (W), *pho1* mutants (P1) and *pho2* mutants (P2) of *Arabidopsis* grown on agar medium with full-strength nutrients. Arrows indicate acid phosphatase activity present in the *pho1* mutant only.

2.4. Summary

In this chapter, total P and Pi concentrations in *pho2* mutants and wild-type plants were measured. *pho2* mutants were shown to accumulate Pi only in their leaves. Zinc deficiency was demonstrated not to be responsible for the Pi accumulation in *pho2* mutants because the Zn concentrations in shoots and roots of *pho2* mutants are similar to those in wild-type plants over a range of growth stages. The localisation of the excess Pi accumulated in *pho2* leaves was studied by measuring the Pi concentrations directly in the protoplasts. The Pi concentrations in protoplasts based on protein, chlorophyll and cell number are consistently greater in *pho2* mutants than in wild type, suggesting that most of Pi in *pho2* leaves resides within the symplast.

The similar activity of acid phosphatase in *pho2* leaves and wild type indicates that *pho2* mutant is not likely to be a mutation in a gene that de-represses the expression of acid phosphatase genes. The *pho2* mutation may specifically affect Pi uptake or transport in the plants.

Chapter 3

Phosphate uptake and translocation in pho2 and wild-type Arabidopsis

3.1. Introduction

Throughout the growth cycle, *pho2* leaves accumulate more Pi than the wild-type leaves (see Fig. 2.3). The greater Pi concentration in *pho2* leaves could be due to a greater uptake rate of Pi from the medium and/or greater translocation from roots.

In a low range of external Pi concentrations, Pi uptake by roots of many species follows Michaelis-Menten kinetics (see Chapter 1). In a number of plant species, the Pi uptake rate has been reported to increase several fold by P deficiency without a change in the K_m (Anghinoni and Barber, 1980; Lee, 1982; Drew et al., 1984; Mimura et al., 1990). By contrast, there is a report demonstrating a decreased K_m without a change in V_{max} under P deficiency (Cartwright, 1972), while in P-deficient roots of tomato, both V_{max} and K_m increased (Jungk, 1974). The changes in kinetic parameters under P deficiency can indicate the types of mechanisms responsible for the increased Pi uptake. In terms of the carrier hypothesis, an increased V_{max} suggests that the number of transporters increases, while a change in K_m implies that the affinity of existing transporters is altered. It would be important to compare the Pi uptake kinetics between *pho2* and wild type to determine if there are differences in V_{max} or K_m between the genotypes.

Phosphate uptake is postulated to be regulated by a negative-feedback mechanism where Pi recycled in phloem regulates Pi uptake by roots (Lefebvre and Glass, 1982; Drew and Saker, 1984; Marschner, 1995). In this mechanism, the concentration of Pi in the phloem is influenced by shoots and can act as a signal to regulate uptake of Pi by roots. If this recycling system is altered, the uptake of Pi by roots may also change. For example, when running into P deficiency, less Pi is retranslocated from shoots to roots resulting in a decline in root Pi concentration, which in turn may activate Pi uptake.

In this chapter, to investigate the Pi uptake capacity and the ability to retranslocate Pi, uptake experiments using ^{32}Pi were conducted under P-sufficient and P-deficient conditions for both *pho2* mutants and wild-type plants. Kinetics of Pi uptake were also studied in the two genotypes. From these studies, the possible mechanisms underlying the high Pi accumulation phenotype of *pho2* mutants are discussed.

3.2. Materials and methods

3.2.1. Plant growth and Pi assay

Seedlings were grown in hydroponic culture as described in Chapter 2.2.1. To study the effect of P deficiency on Pi uptake, plants were transferred to 0.2 mM $CaCl_2$ solution for 1 h to deplete Pi in the apoplasm before being transferred to nutrient solution without Pi for an additional 4 or 7 d. Plants of same ages were used for uptake studies. In this -P nutrient solution, 250 µM KCl was substituted for KH_2PO_4 to maintain K^+ at the same concentration as in the complete nutrient solution. The control plants (wild type and *pho2*) were continuously supplied with complete full-strength solution. Inorganic phosphate in plants was extracted and assayed as described in Section 2.2.2.

3.2.2. ^{32}Pi uptake assays

Plant roots (3-4 plants) were rinsed with distilled water and transferred to 12 ml of uptake medium of the same composition as the nutrient solution, except that ^{32}Pi (4.9×10^3 Bq / µmol Pi, Amersham International, Amersham, UK) was included. After 1, 2, 4 or 8 h of uptake, plants were transferred to 45 ml of the same aerated nutrient solution that lacked ^{32}Pi and allowed to desorb for about 2 min. Shoots and roots were then collected and weighed separately in glass vials. Plant material was ashed in a furnace at 540 °C overnight and after the vials had cooled, 0.5 ml of HNO_3:$HClO_4$ (v:v=9:1) was added to each sample. Plant material was digested further on a hot plate at 90 °C for about 2 h until the samples had just dried. Distilled water (0.6 mL) was added to the vials, the samples were vortexed and to 0.3 ml of the final solution, scintillant (3 mL) was added to determine the

radioactivity by scintillation counting (Beckman LS6800). The scintillant was composed of 0.6 % (w/v) 2,5-diphenyloxazole (AJAX Chemicals Ltd., Sydney-Melbourne, Australia) dissolved in 33.3 % (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) and 66.7 % (v/v) xylene (The British Drug Houses Ltd., Poole, England). In some experiments, the shoots of wild-type and *pho2* plants were cut off immediately prior to transferring the roots to the uptake medium.

3.2.3. Kinetics of ^{32}Pi uptake

3.2.3.1. Pi uptake over a range of external Pi concentrations

To study the kinetics of ^{32}Pi uptake, roots of plants grown in full-strength nutrient solution were rinsed with distilled water and moved to -P nutrient solution for about 10-20 min to deplete the Pi in the apoplast. Plants were then transferred to uptake solutions that were ^{32}P-labelled (4.9×10^3 Bq/µmol Pi) and had KH_2PO_4 concentrations of 1, 2, 4, 8, 16 or 32 µM. These uptake solutions were otherwise identical to those used for plant growth. For each treatment, ^{32}Pi uptake was monitored over 1 h. To keep Pi depletion by plants less than 10% of the total Pi in the uptake solution during the experimental period, larger volumes of uptake solution (1 L) were used for the low Pi concentration treatments (1-4 µM). After uptake, the plants were transferred to non-labelled Pi solution for 5 min to desorb ^{32}Pi adhering to roots. Shoots and roots were harvested separately in pre-weighed vials, and ^{32}Pi activity was analysed by methods described above (3.2.2). The data were best fitted to the Michaelis-Menten equation by estimating the Vmax and Km using a non-linear least squares method. The fitting of data was conducted by running a program (Solver in Excel 5.0) written by Mr. Leon Miguel (University of Western Australia).

3.2.3.2. Kinetics of Pi uptake measured by a solution depletion technique

Plants grown for 26 d (with 250 µM Pi) were transfered to full-strength nutrient solution containing 15.3 µM KH_2PO_4 to deplete excess Pi adhering to the root system. After 1 h, the roots were taken out of the solution and carefully blotted with tissue paper. Plants were

then transferred to uptake medium (12 mL) containing the same concentration of KH$_2$PO$_4$ (15.3 μM) except that ^{32}Pi was included (4.9 × 10^3 Bq/μmol Pi). Subsamples (8 μL) were taken from the uptake medium before plants were transferred and then every 10 min during the uptake period for radioactivity assay as described in 3.2.2. At the end of the experiments, the uptake medium was filtered (0.22 μm pore diameter, Millipore Corp., Bedford, MA, USA), and 50 μL of the filtrate was used for assay of radioactivity.

The Michaelis-Menten equation $\dfrac{-d[C]}{dt} = \dfrac{V_{max}[C]}{K_m + [C]}$ can be solved as a differential equation of two variables, [C] and t. Thus, we obtained:

$$V_{max}t + K_m \ln\left(\frac{[C]}{[C_0]}\right) + [C] - [C_0] = 0$$

where C$_0$ is the initial Pi concentration in uptake medium and C is the concentration in the medium at time t. Km and Vmax can be varied and best fitted to the series of data points for depletion by a non-linear least square method using Solver in Excel 5.0.

Since the plug of rock wool on which plants were grown was dipped into the uptake medium, the possible adsorption of isotope by rock wool was also checked. The rock wool was soaked in nutrient solution with 250 μM Pi, then in 15.3 μM Pi followed by labelled 15.3 μM Pi uptake medium. These solutions were the same as those used for plants. Subsamples of the uptake medium in which rock wool was soaked were taken for radioactivity assay.

3.2.4. Translocation of ^{32}Pi from leaves to roots

Nineteen-day-old wild-type and *pho2* plants were transferred to 1 L nutrient solution with or without Pi. A needle was used to prick a hole in one leaf blade and 2 × 2 μL of 100 mM KH$_2$PO$_4$ labelled with 37 × 10^{-4} MBq (in 2 μL) was applied. Twenty minutes later, 2 μL distilled water was applied to the same site and this was repeated once again. After 4 d, leaves that had the ^{32}Pi applied were detached and discarded. The plants were then carefully spread, covered with cling wrap and the distribution of radioactivity was determined with a PhosphoImager system (Molecular Dynamics).

3.3. Results and Discussion

3.3.1. ^{32}Pi uptake by *pho2* and wild-type plants

Uptake of Pi by *pho2* mutants and wild-type plants was monitored using ^{32}Pi. The uptake rate for both wild type and *pho2* was constant over 8 h, with *pho2* having about a two-fold greater uptake rate than wild type (2.5 and 1.3 µmol/g root fw/h respectively, Fig. 3.1a). During this period, shoots of the *pho2* mutants always contained a much greater proportion of the ^{32}Pi taken up than wild-type seedlings (Fig. 3.1b). This result is consistent with the greater Pi concentration in shoots of *pho2* than wild type and indicates that *pho2* not only had a greater Pi uptake by roots but also translocated more Pi from roots to shoots.

The effect of P deficiency on Pi uptake and transport by wild-type seedlings and *pho2* mutants was also assessed. Figure 3.2a shows that wild-type plants starved of P for 4 and 7 d increased their Pi uptake rate about 4-fold compared to the plants supplied with sufficient P. This is consistent with many other investigations using different plants species (for example, Drew et al., 1984; Goldstein et al., 1988; Bieleski and Läuchli, 1992) and *Arabidopsis* in particular (Dunlop et al., 1997). Phosphate starvation also induced an increase in uptake by *pho2* mutants, but to a lesser extent (about 2-fold). In contrast to P-sufficient plants, P-starved *pho2* mutants had marginally lower Pi uptake rates than P-starved wild-type plants (Fig. 3.2a). The shoot-to-root ratio of ^{32}Pi accumulated during the uptake period was increased by P starvation in both wild-type and *pho2* seedlings, but *pho2* plants always accumulated more ^{32}Pi to shoots than wild type under both plus (+) and minus (-) P conditions (Fig. 3.2b).

To test whether the greater Pi uptake by the *pho2* mutant was due to a greater shoot sink for Pi or due to a greater inherent Pi uptake by the root, I removed the shoots from *pho2* as well as from wild-type plants and compared their Pi uptake. Figure 3.3 shows that there was no difference in Pi uptake between *pho2* and wild-type plants under these conditions. Roots kept taking up Pi from the solution over the period of the experiment but the uptake
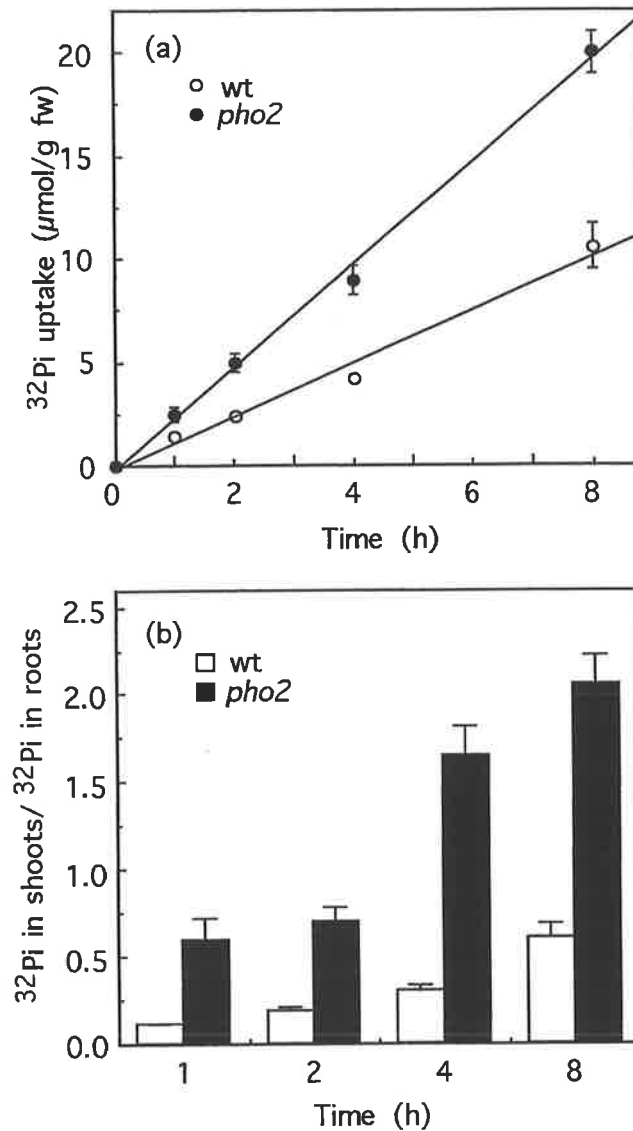
Fig. 3.1. Time-course of Pi uptake (a) and shoot-to-root ratios of the ^{32}Pi taken up (b) by wild type (wt) and *pho2* seedlings over 8 h. Plants were grown with 250 μM Pi in the nutrient solution and uptake was conducted in the same concentration of Pi in the medium for 1, 2, 4, and 8 h. The error bars represent the SE of the mean from five replicates, and the absence of error bars denotes that the errors were smaller than the symbol.
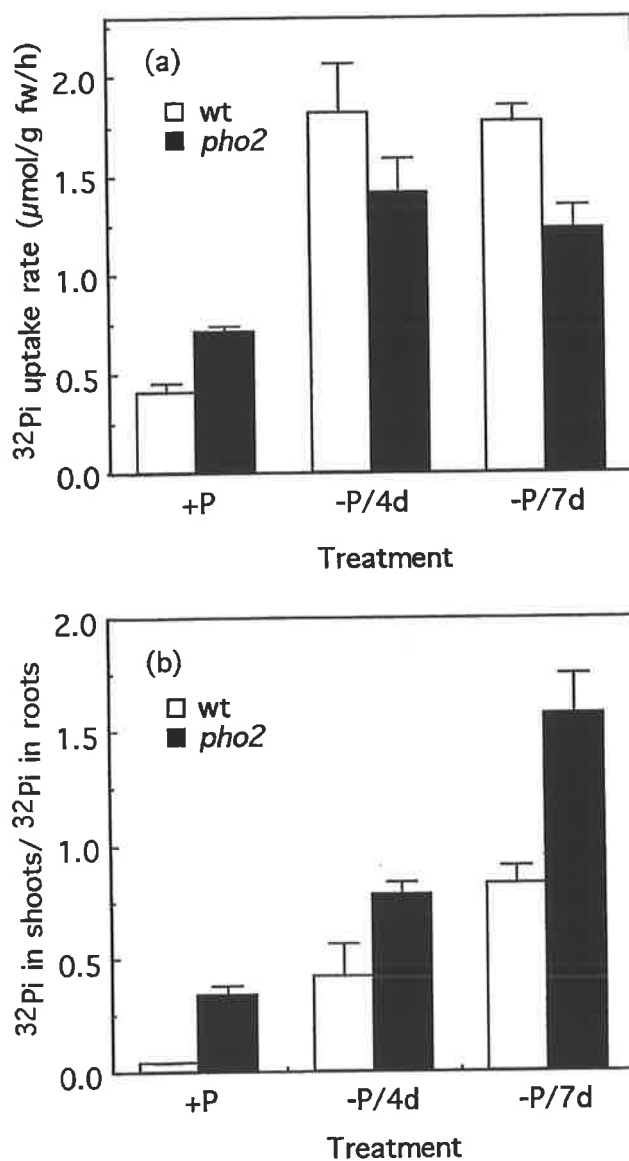
Fig. 3.2. Phosphate uptake rate (a) and shoot-to-root ratio of ^{32}Pi taken up (b) by P-deficient and P-sufficient plants of wild-type (wt) and *pho2* seedlings. Plants were continuously supplied with 250 µM Pi (+P) or deprived of Pi for 4 (-P/4d) or 7 d (-P/7d). The uptake of the same age of plants was conducted with 250 µM Pi in the uptake solution for 1 h. The error bars represent the SE of the mean from four replicates.
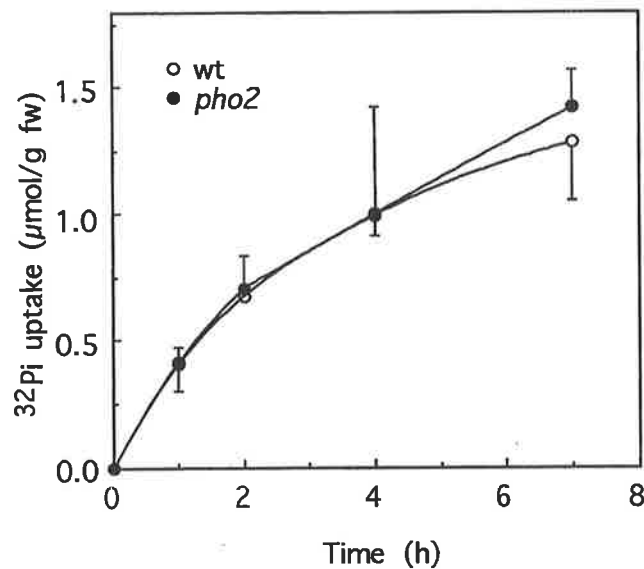
Fig. 3.3. Time course of Pi uptake by wild type (wt) and *pho2* seedlings without shoots. Plants were grown in 250 μM Pi nutrient solution and shoots were removed immediately before being transferred to the same Pi concentration in the uptake solution. The error bars represent the SE of the mean from three replicates.

rate declined after about 2 h (Fig. 3.3). Compared with intact plants, [32]Pi uptake rate of "shootless" plants was reduced over 7 h (Fig. 3.3 and Fig. 3.1a) but over the first hour, uptake was similar in "shootless" plants (0.4 μmol/g root fw/h, Fig. 3.3) to intact wild-type plants (0.4 - 1.2 μmol/g root fw /h in Figs. 3.1 and Fig. 3.2).

It is possible that removing the shoot itself affects root Pi uptake such that inherent differences found in roots of intact plants of the different genotypes are not apparent in the shootless state. To examine this, another experiment was conducted using only wild-type plants grown in +P and -P for 4 d. Shoots of these wild-type plants were removed and [32]Pi uptake was monitored over 1 h. The [32]Pi uptake rate by these plants is shown in Table 3.1. The minus P treatment still enhanced the [32]Pi uptake rate by approximately 2.5-fold over the +P treatment indicating that at least within 1 h, the difference in Pi uptake by shootless plants due to the treatments still existed. This suggests that the method used for measuring Pi uptake in "shootless" *pho2* and wild-type plants is reliable.

Table 3.1. ^{32}Pi uptake rate over 1 h by shootless wild-type *Arabidopsis* with different Pi treatments.

Treatment	^{32}Pi uptake rate (μmol Pi/g fw/h)
+P	0.12 ± 0.01
-P	0.29 ± 0.01

Although the Pi uptake rate for intact *pho2* plants was consistently greater than for wild type within an experiment, rates differed between experiments (for example, see Fig. 3.1a and 3.2a). The experiment of Fig. 3.1 was conducted under a higher light intensity (35 μmol photons m^{-2} s^{-1}) than the experiment shown in Fig. 3.2 (7 μmol photons m^{-2} s^{-1}) and hence caused a greater transpiration rate where a fan was used to cool the room. Delhaize and Randall (1995) showed that the severity of P toxicity in the *pho2* mutant is related to the transpiration rate. The higher Pi uptake rate shown in Fig. 3.1a is consistent with a higher transpiration rate increasing Pi concentrations in the *pho2* leaves through an enhanced uptake and translocation.

Usually the capacity of plant roots to take up Pi increases when plants are deprived of P and decreases with an increase in the internal concentration (Clarkson and Scattergood 1982; Cogliatti and Clarkson 1983; McPharlin and Bieleski 1989). Under P-sufficient conditions, *pho2* has a greater uptake rate than wild-type plants and accumulates Pi to shoots beyond physiological requirements. This suggests that the mechanism that regulates Pi uptake in *pho2* is disrupted. Since Pi concentrations in roots of wild type and *pho2* were equivalent (Fig. 2.3b in Chapter 2), the increased Pi uptake in *pho2* is more likely to be due to the shoots providing a continuous sink for Pi. This was confirmed by experiments where shoots were removed, resulting in a disappearance of the differences in root Pi uptake rate between wild-type and *pho2* seedlings (Fig. 3.3). The observation that the shoot-to-root ratio of ^{32}Pi taken up from the medium was always greater in *pho2* than wild-type plants also indicates a greater sink for Pi in shoots of the *pho2* mutant (Fig. 3.1b and Fig. 3.2b). Further studies on Pi uptake by protoplasts isolated from leaves and roots of *pho2* and

wild-type seedlings were conducted in an attempt to compare their ^{32}Pi uptake rate. Unfortunately, *Arabidopsis* protoplasts isolated by methods described for other plant species seemed to be too fragile and no reliable data were obtained.

Although Pi uptake was greater by *pho2* mutant than wild type under P-sufficient conditions, the Pi uptake rate of both *pho2* and wild type increased to about the same level when P-deficient (Fig. 3.2a). This indicates that roots of *pho2*, when P-sufficient, do not have maximal Pi uptake rates and do not behave as if they are "functionally P-deficient". The activity of acid phosphatase in *pho2* mutants being similar to that in wild type as described in Chapter 2 also illustrates the point that *pho2* is unlikely to be a generally derepressed mutant.

3.3.2 Kinetics of Pi uptake by *Arabidopsis*

In the kinetic studies on *Arabidopsis* presented here, the high-affinity system for Pi uptake, which is usually dominant under low external Pi concentrations, was of primary interest and was studied using two methods.

First, the Pi uptake rate was examined over external Pi concentrations ranging from 1 to 32 μM. The Pi uptake rate was saturated within this range of Pi concentrations with a Vmax of 490 nmol/g fw/h for wild type and 950 nmol/g fw/h for *pho2* mutants (Fig. 3.4). This is consistent with the above results where *pho2* has a greater Pi uptake capacity than wild-type seedlings (Fig. 3.1a). Since the data were quite variable with relatively poor $R^2=0.43$ for wild type and $R^2=0.63$ for *pho2* plants, the Km could not be reliably estimated in this experiment. Although the affinity parameter Km could not be obtained, it appeared to be less than the lowest Km of 2.5 μM previously reported for suspension-cultured tobacco cells (Shimogawara and Usuda, 1995). The reason for the large variation of the data is not known and repeated experiments did not yield a reliable Km. The small and delicate root system of *Arabidopsis* which is difficult to handle may account for this. In addition, the stirring of the uptake medium may not have been consistent for all samples and may have affected the accuracy of the uptake measurements.
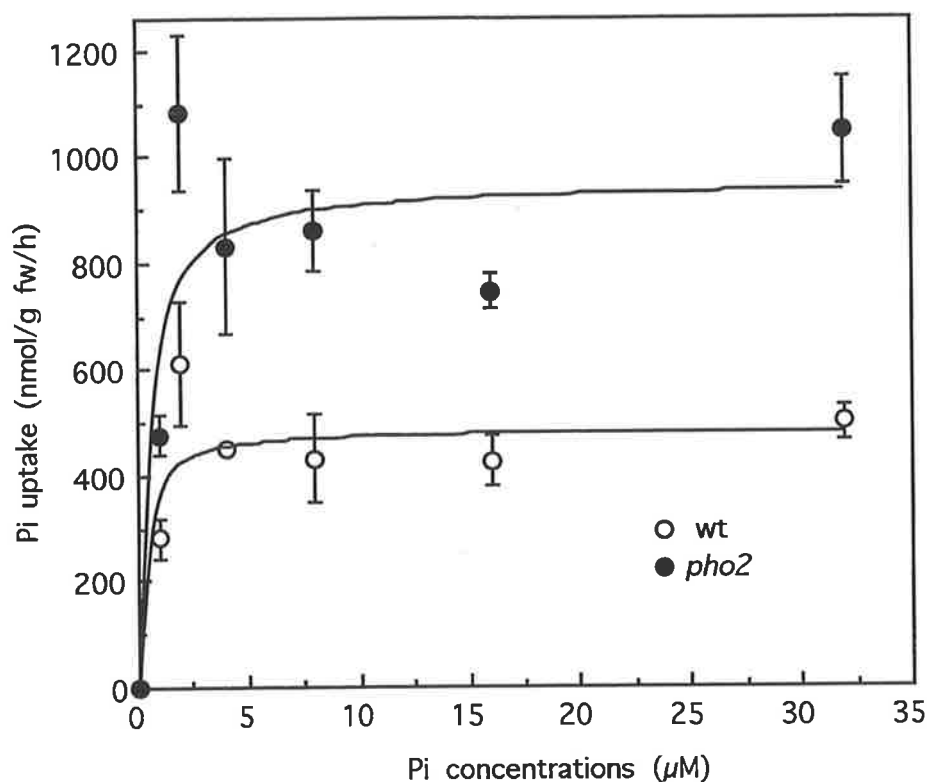
Fig. 3.4. Kinetics of Pi uptake by wild-type (wt) and *pho2* mutant of *Arabidopsis*. Plants were grown in 250 μM Pi solution for 26 d; after the roots were rinsed with -P nutrient solution, plants were transferred to the solutions containing 1, 2, 4, 8, 16 or 32 μM ^{32}Pi. Uptake was conducted for 1 h in each ^{32}Pi concentration. The error bars represent the SE of the means from 3 to 4 replicates.

A disadvantage of the above method is that different plants were used for each concentration and differences between the individual plants may have also contributed to the variability of the data. In addition, for the Michaelis-Menten equation, theoretically the substrate concentrations (C) is assumed not to change over the uptake period, while this assumption is valid for high Pi concentrations where depletion of Pi over 1 h is negligible, depletion of Pi at the low Pi concentrations (e. g, 1 μM) may have been significant.

A method based on Pi depletion overcomes some of the disadvantages mentioned above. Classen and Barber (1974) monitored the depletion of nutrient from solutions to determine

kinetic parameters by fitting a curve to the data with a least squares procedure. The experiment can be conducted with the same set of plants which eliminates errors associated with variability between individual seedlings. Figure 3.5 (from Drew et al. 1984) shows a theoretical time-course of Pi depletion by roots in a constant volume of uptake medium using four combinations of Vmax and Km. In each case, the decline in nutrient concentration is initially near-linear and then gradually decreases as the concentration (C)
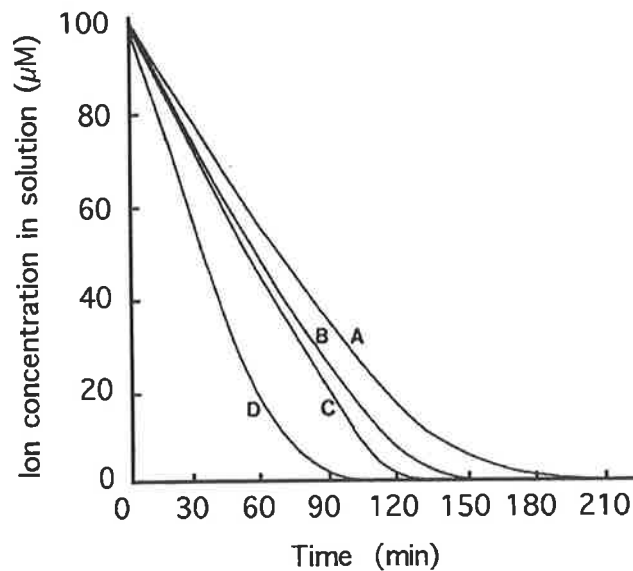
Fig. 3.5. Theoretical relation between ion concentration remaining in uptake solution and time during uptake by plant roots in low external ion solution. Values of Km (μM) and of Vmax (nmol/g/min) were as follows: A, Km=20, Vmax=1; B, Km=10, Vmax=1; C, Km=5, Vmax=1; D, Km=20, Vmax=2. The solution volume was 1 litre. From Drew et al. (1984).

approaches zero with time. The initial slope of the curve is determined by Vmax and the later part of the curve by Km. Estimation of low Km values depends on accurate measurement of C when it is near zero. In my experiments, I examined 26-d-old *Arabidopsis* of wild type and *pho2* seedlings depleting Pi in uptake medium against time as shown in Fig. 3.6. However, the data did not fit the Michaelis-Menten equation. The rapid decrease of the Pi concentration at the first time point would indicate that the concentration used might not have been high enough to saturate the transporters (Fig. 3.6). However,

even higher starting concentrations (from 20 to 40 µM) produced similar types of curves, with considerably quicker Pi depletion in the first few time points than later ones (data not shown). One problem when using the higher starting Pi concentrations is that the Pi concentration could not be sufficiently depleted within several hours. With the small volume of uptake solution, evapotranspiration over several hours becomes significant and will cause higher estimates of the Pi concentrations especially for the later points when the Pi concentrations are very low.
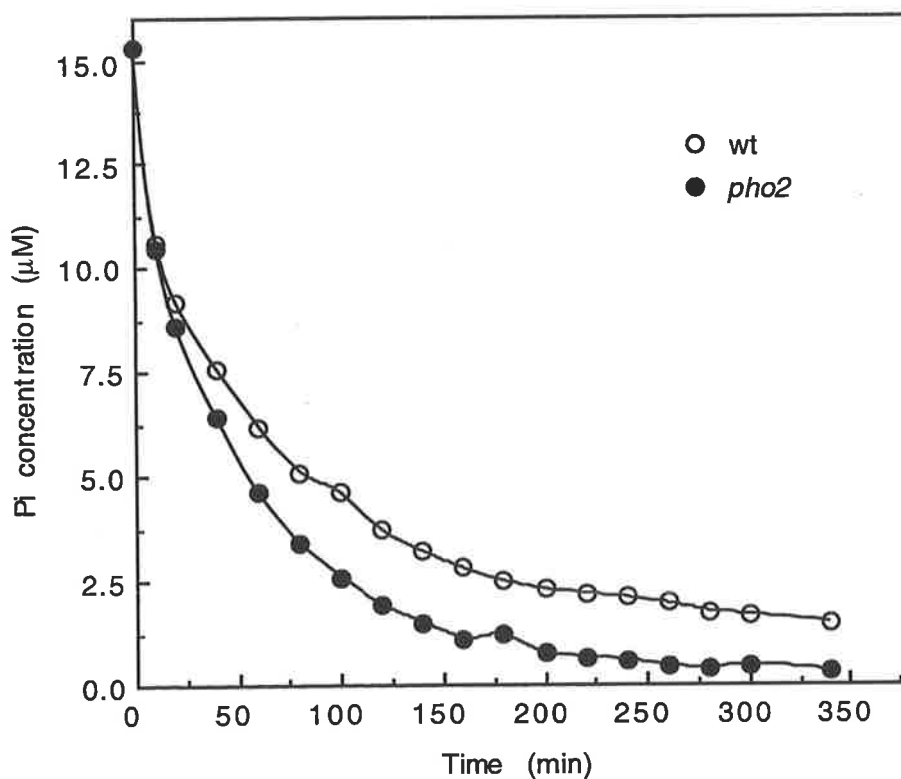
Fig. 3.6. Depletion of Pi by wild-type (wt) and *pho2 Arabidopsis*. Plants grown in nutrient solution containing 250 µM Pi were transferred to 12 ml of uptake medium containing 15.3 µM Pi. These are typical curves from five experiments.

According to the Michaelis-Menten kinetics, Pi concentration in medium should finally reach zero. The failure of roots to reduce the Pi concentration of labelled Pi in uptake medium to zero but to a minimum concentration of Cmin was taken as evidence of an appreciable efflux component (Claassen and Barber, 1974; Anghinoni and Barber, 1980). It

may also mean that transporters have some threshold before they start working. I examined the labelled uptake medium by comparing radioactivities of the filtered uptake medium with the uptake medium at the end of the experiment. Radioactivity in the filtered solution was about half of that in the unfiltered uptake medium, indicating the remaining radioactivity was not all in free solution but associated with particles that might include microbes and root-cap cells detached by the aeration.

Radioactivity in the uptake medium with rock wool was also examined to see whether the wool itself would absorb isotope and affect the measurements. Table 3.2 shows that over 2 h, there was no significant decrease in radioactivity in the uptake medium, suggesting that rock wool was an inert material appropriate for the experiments.

Table 3.2. Effect of rock wool on the radioactivity of ^{32}Pi and Pi concentration in the uptake medium. Subsamples of 12 μl were used for assay.

Time (h)	0	0.5	1	1.5	2
cpm	6254	6136	6194	6199	6124
Pi concentration (μM)	15.3	15.0	15.2	15.2	15.0

Although the kinetic studies were not fully successful, they provided evidence that *pho2* does have greater Pi uptake capacity over a range of Pi concentrations and that the Km of *pho2* does not appear to be greatly different from that of the wild-type plants.

3.3.3. Translocation of ^{32}Pi from shoots to roots

Previously Delhaize and Randall (1995) showed that most of the P in *pho2* can be mobilised out of a developed leaf but the fate of this P was not known because radiolabelled Pi was not used in those experiments. To compare the capacities of *pho2* mutants and wild-type *Arabidopsis* to translocate Pi from their leaves, I applied ^{32}Pi on a single leaf and observed that both wild type and *pho2* plants can translocate Pi to other leaves as well as from leaves to roots under both P-sufficient and P-deficient conditions (Fig. 3.7).

wild-type   pho2

-P

+P
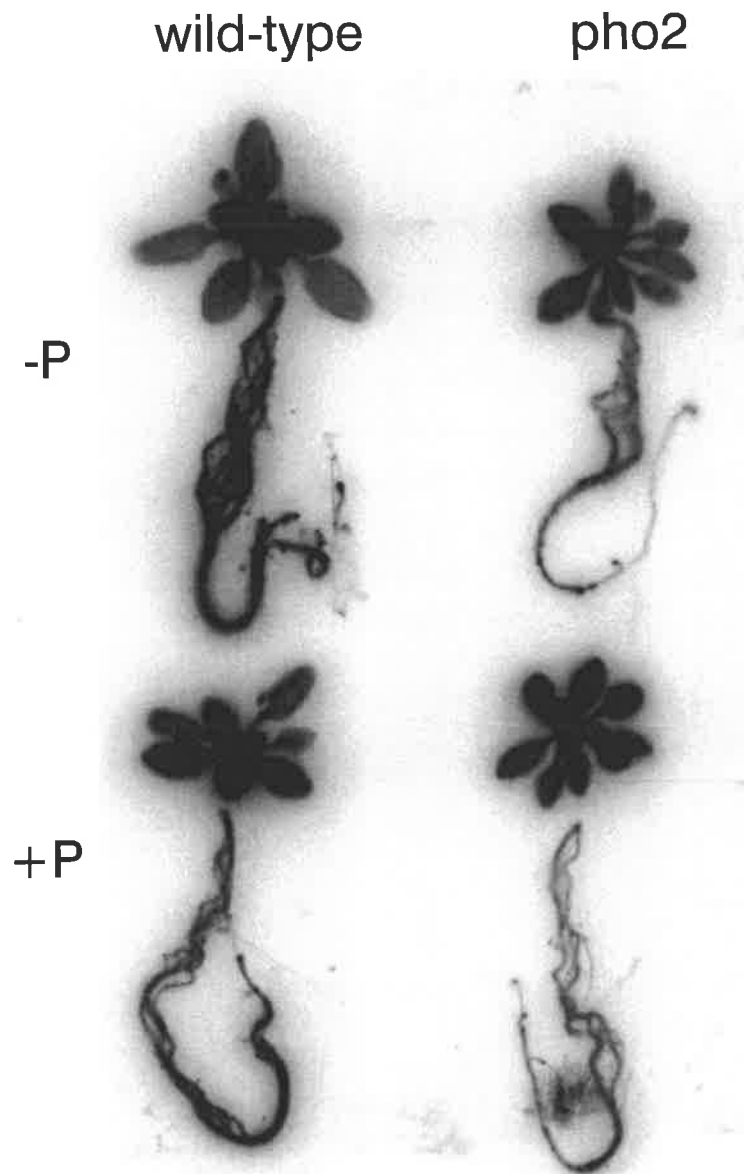
Fig. 3.7. Translocation of ^{32}Pi from a single leaf to other plant parts of P-deficient and P-sufficient seedlings of wild type (wt) and *pho2* mutant. Plants grown in 250 µM Pi nutrient solution were moved to the same Pi concentration solution (+P) or nutrient solution without Pi (-P) when ^{32}Pi was applied on one of the leaves of each plant. Plants were grown in these solutions for an additional 4 d before the leaf to which the label was applied was removed and the plants were analysed using a PhosphoImager.

However, the proportion of ^{32}Pi translocated from shoot to root was 2-fold greater for wild-type plants than for the *pho2* mutant under +P conditions and about 2.5-fold greater under -P conditions (Table 3.3).

Table 3.3. Percent distribution of ^{32}Pi between shoots and roots after translocation from a single ^{32}P-labelled leaf. The activity of ^{32}P was quantified by a PhosphoImager system.

genotypes	+P		-P	
	shoot	root	shoot	root
wild-type	67±4% [a]	33±4%	55±4%	45±4%
pho2	85±4%	15±3%	83±1%	17±1%

[a] mean±standard error. n=3.

Since the amount of ^{32}Pi absorbed by leaves after application of the radiolabel may have been variable and also the specific radioactivity of ^{32}Pi in *pho2* and wild type leaves were different due to different Pi concentrations in the leaves, the actual amount of Pi translocated to roots could not be determined in this experiment. To assess the ability of *pho2* to translocate Pi to roots, plants were grown with a sufficient supply of Pi and then transferred to nutrient solutions that lacked Pi. The Pi concentrations of shoots and roots were then monitored over 7 d to determine whether or not *pho2* is capable of retranslocating Pi from shoots to maintain root Pi concentrations. At the start of the experiment, the Pi concentration in shoots of *pho2* was about 4-fold greater than that of wild-type seedlings, while the Pi concentrations in roots were similar (Fig. 3.8). When the plants were deprived of external Pi, the Pi concentrations in shoots of wild type and *pho2* decreased with time, although *pho2* always maintained about a 4-fold greater concentration than wild type. By the seventh day of starvation, Pi concentrations decreased to about one-fifth of that measured on day 0 for the control plants; in *pho2*, the concentration was about 0.5 µg/mg fw which is similar to the concentration in the P-sufficient wild-type seedlings. By contrast, the Pi concentration in roots was depleted at a similar rate for both *pho2* mutant and wild-type plants. By the seventh day of starvation, both genotypes had reached the same final Pi

concentration in roots, which was about one-tenth of the initial Pi concentration. The decrease in Pi concentrations in both genetypes is more likely to be due to Pi being incorporated to organic P in the developing tissues rather than a "dilution effect" because the plant growth within 7 d did not increase 5- to 10-fold.
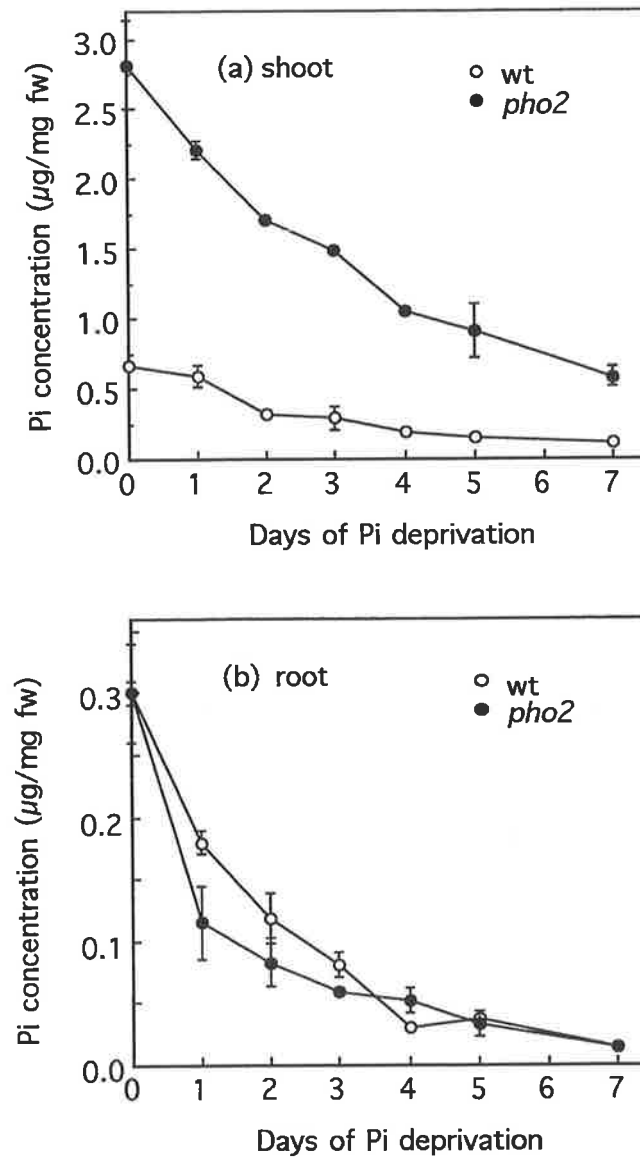
Fig. 3.8. Phosphate concentrations in shoots (a) and roots (b) of wild-type (wt) and *pho2* seedlings running into P deficiency. Plants grown in 250 μM Pi nutrient solution for 26 d were moved to nutrient solution without P, and Pi concentrations in the plants were monitored over 7 d. The error bars represent the SE of the mean from three replicates when larger than the symbols.

These results are consistent with the other experiments where shoot-to-root distribution ratios of ^{32}Pi were always greater for *pho2* than wild-type plants regardless of whether plants were P-sufficient or P-deficient, suggesting that the recycling mechanism from shoots to roots in *pho2* is partially defective. Although the Pi concentration in *pho2* shoots was about 4-fold greater than that of wild type throughout the experiment (Fig. 3.8), *pho2* did not appear to translocate this excess Pi to roots when P-deficient and appears to "hold" Pi in shoots, resulting in a similar depletion of Pi concentration in its roots as in wild-type plants. A reduced flow of Pi from shoots to roots in the *pho2* mutant may be responsible for the enhanced Pi uptake by roots.

3.4 Summary and Conclusion

In this chapter, several experiments on Pi uptake and translocation were conducted by comparing *pho2* mutant and wild-type plants. The greater Pi uptake capacity of *pho2* mutants appears to be due to a greater sink for P in shoots. The *pho2* shoots are able to re-translocate Pi to other parts of the plant but the amount of Pi translocated from shoots down to roots was less than in wild-type plants. Phosphate deficiency further increased the Pi uptake by *pho2* mutants to a similar level of wild-type plants indicating that the *pho2* mutant is not "functionally P-deficient".

Two possible mechanisms are proposed to explain the phenotype of the *pho2* mutant: 1) decreased export of Pi from shoot cells to root cells; or 2) enhanced uptake by shoot cells of Pi supplied by the xylem. In the first case, since the translocation of Pi from older leaves to young leaves of the *pho2* plants was found to be normal when plants were running into P deficiency (Delhaize and Randall, 1995), the blockage in phloem transport of *pho2* mutants is not general. The ^{32}Pi translocation and Pi depletion experiments presented in this chapter provide evidence that Pi transport in phloem from shoots to roots in *pho2* mutants could be specifically affected. The smaller proportion of Pi re-translocated down to roots in *pho2* mutants may be due to mutation in a gene that does not function effectively and causes shoot cells to build up a greater Pi concentration than in wild-type plants. However, since the intact *pho2* mutants have greater Pi uptake capacity than wild-type plants, it appears that

pho2 shoots act as the "driving force" for Pi uptake and that the second proposed mechanism could also result in Pi accumulation. At the molecular level, a gene encoding a Pi uptake regulator could be mutated, or a Pi transporter on either the plasmallema or tonoplast of shoot cells could be overexpressed regardless of sufficient Pi in cells, and therefore, resulting in excessive accumulation of Pi in *pho2* shoot cells. Excessive Pi uptake by shoot cells could then indirectly influence root Pi uptake by enhancing Pi flux to the xylem. An uncontrolled uptake of Pi by shoot cells might also explain the observed greater sink for Pi in *pho2* shoots and the relatively poor ability of *pho2* to translocate Pi from shoots to roots. Furthermore, within the shoot cells of *pho2*, the distribution of Pi between cytosol and vacuole could be disturbed. For example, excess Pi translocated to vacuoles by an overactive transporter on the tonoplast could continously deplete cytoplasmic Pi concentrations which in turn results in greater Pi uptake at the plasmallema.

Chapter 4

Phosphate transporter genes of Arabidopsis

4.1 Introduction

Although the physiology of phosphate uptake has been extensively studied, knowledge regarding the molecular basis for Pi uptake is still lacking. To understand the nature and mechanisms of Pi transport in plants, the isolation and characterisation of the transporter proteins is necessary. However, the abundance of nutrient transporters is likely to be very low, and direct isolation of membrane transporters is difficult. Except for some relatively abundant membrane proteins, such as H^+-ATPase, no successful large scale isolation of plasma membrane nutrient transporters has been achieved (Clarkson and Hawkesford, 1993). Rather than purifying transporters directly, the isolation of genes encoding these transporters and their expression in heterologous systems is an alternative approach.

Since the isolation of a high-affinity Pi transporter gene (*PHO84*) from *Saccharomyces cerevisiae* using the *pho84* mutant (Bun-ya et al., 1991), cloning and characterisation of homologous genes from other species such as the filamentus fungus *Neurospora crassa* (Versaw, 1995) and the mycorrhizal fungus *Glomus versiforme* (Harrison and van Buuren, 1995) were also successful. In higher plants, through the use of an expressed sequence tag (clone VBVIF01, accession no. Z337763) from *Arabidopsis* whose translation product resembles yeast Pho84p, two phosphate transporter genes were isolated by Smith and colleagues at the CSIRO Division of Tropical Agriculture, Queensland. These genes were named *APT1* and *APT2*. Although the coding regions of the two genes are almost identical, the 5' region upstream of the start codon are very different. The two genes encode polypeptides of 524 amino acids and by comparison with protein sequences on the databases (Altschul et al., 1990), the proteins exhibit about 37 % identity with Pho84p of yeast and 43 % identity with GvPT, a phosphate transporter whose gene was cloned from *G. versiforme*. The deduced structure of the proteins is predicted to contain 12 membrane-

spanning domains with a 6+6 pattern which has been found to be a typical structure for membrane transport proteins (Marger and Saier, 1993).

Since _pho2_ and _pho1_ could result from mutations in Pi transporter genes, it was of interest to determine whether _APT1_ or _APT2_ encode _PHO1_ or _PHO2_, or if the _APT_ genes are regulated by either _PHO1_ or _PHO2_. In this chapter, I describe my contribution to the characterisation of the _APT1_ and _APT2_ genes (referred to as _APT1/2_) in a collaboration with Dr. Frank Smith's group. Specifically, experiments aimed at mapping the genes on the _Arabidopsis_ genome and relating their expression to the P nutrition of wild-type plants and _pho2_ mutants are described.

4.2. Materials and methods

4.2.1. Plant growth

Arabidopsis thaliana seedlings were grown in hydroponic culture as described in Section 2.2.1. For the P-starved treatment, KH_2PO_4 was omitted and KCl (250 μM) was added to maintain the concentration of K^+ ions. Similarly, for the N-starved treatment, KNO_3 was replaced with 250 μM KCl and for the K-starved treatment, KNO_3 and KH_2PO_4 were replaced with 625 μM $NaNO_3$ and 250 μM NaH_2PO_4. In some experiments, plants were starved of Pi for 7 d and then resupplied with full-strength nutrient solution containing 250 μM KH_2PO_4. Seeds to be grown in soil were planted in pots that contained potting mixture high in composted organic matter. The pots were kept at 20 °C until harvest.

4.2.2. Genomic DNA extraction and Southern blot analysis

Genomic DNA was extracted from _Arabidopsis_ ecotypes Columbia (Col) and Landsberg _erecta_ (L._er_) plants grown in soil. Shoots were ground in liquid nitrogen and the powder was extracted with cetyltrimethyl ammonium bromide buffer and DNA was purified with $CsCl_2$ gradient centrifugation (Murray and Thompson, 1980). After centrifugation, the DNA layer was removed from the tube using a 2 ml syringe with an 18 gauge needle and

further extracted with phenol/chloroform. The ethanol-precipitated DNA pellet was finally

resuspended in TE buffer.

To screen for restriction fragment length polymorphisms (RFLPs), genomic DNA extracted

from Col and L.*er* were digested with each of 10 restriction endonucleases (Bresatec) at 37

°C overnight. The mixture contained 20 μL enzyme buffer (10 × buffer provided by

supplier); 0.5 μL RNAase (10 mg/mL); 3 μl restriction enzyme (10 u/μL); about 3 μg DNA;

and was made up to 200 μL with distilled water. After digestion, DNA was precipitated

with 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol at -20 °C for 1 h.

The sample was centrifuged (13,000 × *g* for 15 min) and the resulting pellet was washed

with 70 % ice-cold ethanol and then air-dried.

Restricted DNA was fractionated on 0.7 % agarose gels. Lamda phage restricted with

*Hind*III was used as the DNA size markers. After electrophoresis, the gel was denatured in

NaCl/NaOH (1.5 M/0.5 M) for 40 min and then rinsed with distilled water. The gel was

then blotted onto a nylon filter (Hybond N, Amersham, UK) with 20 × SSC overnight

using a standard capillary transfer protocol (Sambrook et al., 1989). The filter was rinsed

with 2 × SSC, fixed under UV light for 2 min and pre-hybridised in a solution described by

Khandijian (1987) at 42 °C with denatured salmon-sperm DNA (100 μg/mL). The filter was

then hybridised with a ^{32}P-labelled *APT1* cDNA probe prepared using a Gigaprime DNA

Labelling Kit (Bresatec). After hybridisation, the filter was washed sequentially with 2 ×

SSC, 0.5 × SSC and 0.2 × SSC (with 0.5% SDS respectively) at 65 °C for 20 min each

wash and then placed against a PhosphoImager (Molecular Dynamics) screen or X-ray film

for exposure. For sequential hybridisations, the filter was stripped with boiling 0.1 × SSC

(0.1 % SDS) and shaken for 20 min at room temperature before being reprobed.

To map the *APT1* and *APT2* genes, fifty recombinant inbred lines derived from a Col by

L.*er* cross were obtained from the Arabidopsis Biological Resource Centre (Ohio State

University, Ohio, USA) and the isolated genomic DNA was digested with *Hind*III.

Southern blots of the digested DNA were hybridised with probes prepared from *APT1*

cDNA and 5' regions upstream of the coding regions of *APT1* and *APT2*. Plants were

scored as having either a Col or L.*er* pattern and the map position was determined using the program "Mapmaker" version 1.0 (Lander et al., 1987; Lister and Dean, 1993).

4.2.3. Northern blot analysis

4.2.3.1. Total RNA extraction

Total RNA was isolated from roots, leaves, flowers or siliques using a previously described method (Chandler et al., 1983) with modifications. Briefly, about 0.5 g of plant material was snap-frozen in liquid nitrogen and extracted with buffer containing 0.1 M NaCl, 10 mM Tris.Cl (pH 8.0), 1 mM EDTA and 1% SDS. The mixture was then extracted with phenol/chloroform and centrifuged in a swinging rotor (Sorvall RC 5C, DuPont, USA) at $4,000 \times g$. The nucleic acids in the supernatant were precipitated with sodium acetate and ethanol at -20 °C overnight followed by centrifugation ($13,000 \times g$ for 15 min). The pellet was dissolved in water and precipitated with LiCl (2 M in the final concentration) at 4 °C overnight. After centrifugation, the pellet was resuspended and RNA was precipitated with sodium acetate and ethanol. The final pellet was dissolved in water to a concentration of approximately 2 µg/µL as determined by absorbance at 260 nm assuming that one unit of absorbance is equivalent to 40 µg/mL RNA (Sambrook et al., 1989).

4.2.3.2. RNA gel and Northern blot analysis

Samples of RNA (up to 10 µg) were separated on 1.5% w/v formaldehyde agarose gels. After electrophoresis, RNA was transferred to a nylon membrane (Hybond N, Amersham) using standard methods (Sambrook et al., 1989). The filters were pre-hybridised and hybridised with ^{32}P-labelled *APT1* cDNA probes as described (Section 4.2.2). A control probe, elongation factor 4A (*elF*4A; Taylor et al., 1993), was used to take into account different RNA loadings. The intensity of the bands was quantified using the PhosphoImager system.

4.2.3.3. Mini preparation of RNA control probe *elF*4A

A plasmid containing *elF*4A was kindly provided by Dr. Christie Howard from Michigan State University. The plasmids were electro-transformed into *E. coli* (DH-5α). Mini preparation of the plasmid from *E. coli* was conducted using the alkaline lysis method described by Sambrook et al. (1989). The plasmid was digested with *Bam*HI and *Xho*I and the *elF*4A fragment of about 0.42 kb (Owttrim et al., 1991) was fractioned on a 1% agarose gel and purified using a QIAEX DNA Gel Extraction Kit (QIAGEN, Australia).

4.2.4. Pi concentration and ^{32}Pi uptake assays

Inorganic phosphate concentrations in plants and ^{32}Pi uptake rates were measured as described in Sections 2.2.2 and 3.2.2.

4.2.5. Electrophysiological measurement of Pi uptake

Plants in hydroponic culture were transferred to 0.2 mM CaCl$_2$ for 0.5 h to deplete Pi adhering to roots before measuring the membrane potential. Roots were mounted horizontally in a Perspex chamber (6 ml volume) with a flowing nutrient solution (pH 6.0) that lacked Pi and were left to equilibrate for 30-60 min. Measurements of membrane potential (Em) was made with a borosilicate glass microelectrode (Clark Electromedical Instruments, Reading, UK) filled with 2 M KCl and connected to an Ag-AgCl half-cell. A manually operated micromanipulator (Narishige, Japan) was used to insert the electrodes into the roots (Fig. 4.1). The typical Em for root cells varied between -130 to -180 mV. When a stable Em was obtained, plant roots were then supplied with one of the Pi concentrations that ranged from 0 to 50 μM. Membrane potential was measured with an electrometer amplifier (FD 223, World Precision Instruments, USA) and recorded on a chart recorder. The data were fitted to a Michaelis-Menten equation using the Origin^{TM} computer software.

4.3. Results

4.3.1. Southern blot analysis of the *APT* genes
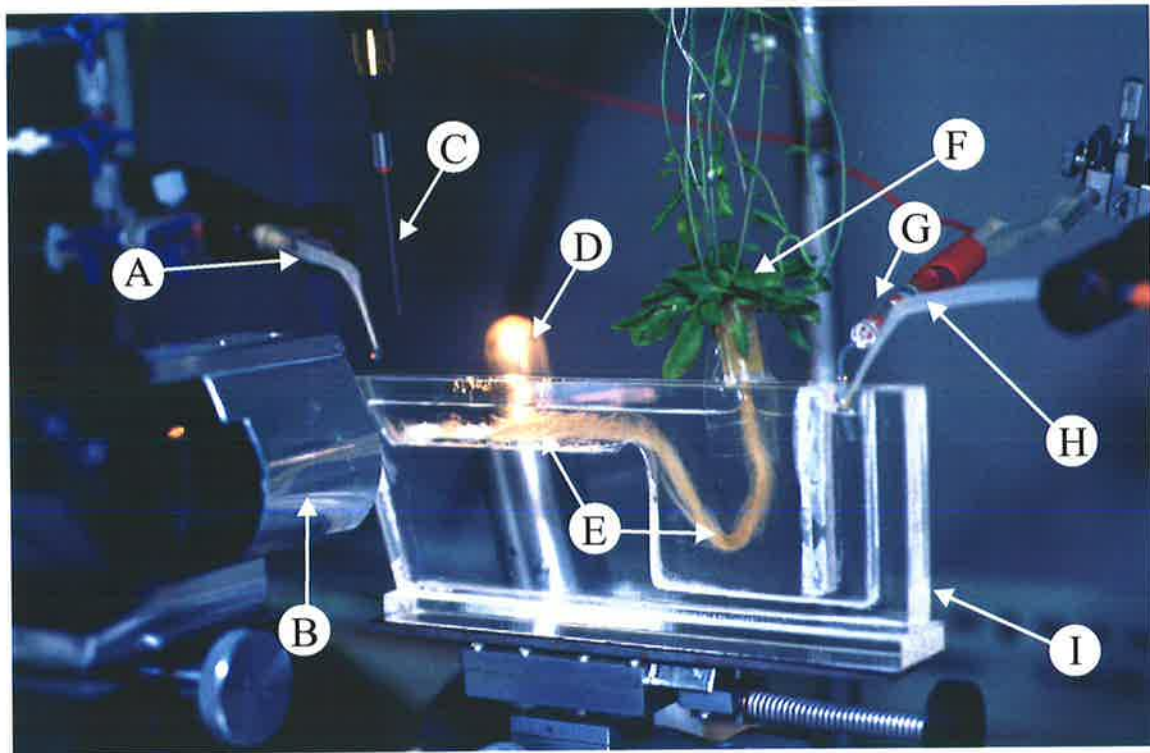
Fig. 4.1. Apparatus used to measure the electrical membrane potential of plants root cells. (A) -- Solution inflow; (B) -- Microscope; (C) -- Glass microelectrode used to impale root cells; (D) -- Light source; (E) -- Plant roots held in solution inside the perspex chamber; (F) - - *Arabidopsis* plant sitting in the chamber; (G) -- Bath electrode; (H) -- Suction to remove solution; (I) -- Perspex chamber for measuring the membrane potential difference in root cells of intact plants.

Southern blot analysis using the coding region of *APT1*, which will cross-hybridise to *APT2*, and gene-specific probes comprising of a region 5' upstream of the coding regions for each gene were undertaken to determine the number and location of the genes. Digestion of genomic DNA with a range of restriction enzymes and subsequent probing with *APT1* cDNA resulted in a banding pattern consistent with the presence of a small gene family of two or more homologous genes in the genome (Fig. 4.2a). However, digestion of Col or L.*er* DNA with either *Hind*III or *Xho*I resulted in a single major band suggesting that the coding regions of *APT1* and *APT2* are genetically linked. Other sequences in the genome related to the *APT1/2* coding regions were inferred from the presence of lower intensity bands in several of the restriction digests. Hybridisation with gene-specific probes simplified the banding pattern and showed the presence of a single gene for each of *APT1* and *APT2* in both Col and L.*er* genomes (Fig 4.2b and c). Both of these probes showed the same RFLP with *Hind*III as observed when the coding region was used as a probe. These data suggest that *APT1* and *APT2* are physically linked and are located on a fragment of 6 kb when Col genomic DNA is digested with *Hind*III.

Using the RFLP defined by a *Hind*III digestion of Col and L.*er* DNA, genomic DNA from fifty recombinant inbred lines was probed with *APT1* cDNA. Figure 4.3 shows an example of a Southern blot using 17 recombinant inbred lines that were hydridised with an *APT1* probe. Gene-specific probes prepared from the 5' upstream of the coding regions were also used to score the patterns of the recombinant inbred lines and they independently mapped to the same locus on chromosome 5 between the markers g4028 (4.6 cM 'south') and m435 (20.6 cM 'north') (Fig. 4.4) confirming that *APT1* and *APT2* are physically linked.
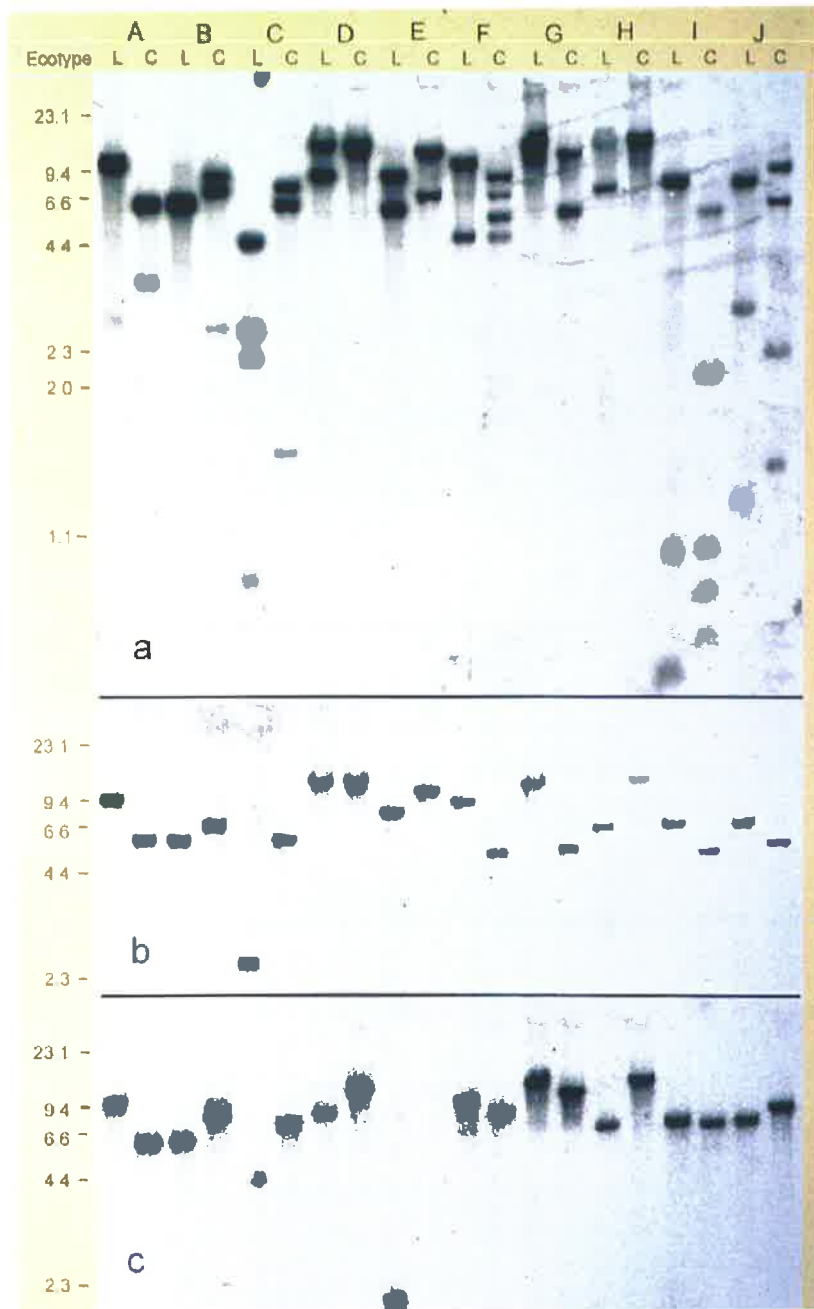
Fig. 4.2. Southern blot analysis of Col (C) and L.*er* (L) genomic DNA (3 µg/lane) digested with 10 different restriction enzymes (A = *Hind*III; B = *Xba*I; C = *Bgl*II; D = *Sal*I; E = *Sac*I, F = *Nco*I, G = *Pst*I; H = *Xho*I, I = *Eco*RV; J = *Bam*HI). The blot was hybridised with *APT1* cDNA probe (a, internal restriction sites in the coding regions and introns are *Bam*HI-one site; *Eco*RV-two sites; *Nco*I-one site; *Sac*I-one site), a gene-specific probe consisting of a 413 bp region immediately upstream of the *APT1* coding region (b) and a gene-specific probe consisting of a 654 bp region immediately upstream of the *APT2* coding region (c). The size markers are bacteriophage λDNA digested with *Hind*III.
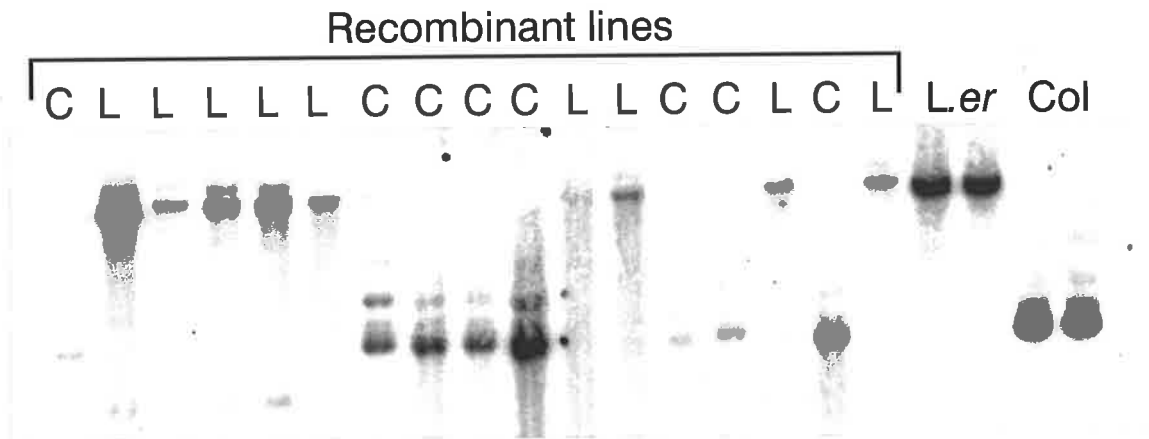
Fig. 4.3. The *APT1* cDNA was used to probe DNA from 17 recombinant inbred lines digested with *Hind*III. The RFLPs defined by the *Hind*III digestion are shown to be derived from the parental lines of Columbia ("Col") and Landsberg *erecta* ("L.*er*"). The hybridisation patterns of the recombinants were scored as "C" for Columbia type or "L" for Landsberg type.

Distance (cM)	Marker Name
	g3715
4.7	m217
1.0	g3837
5.5	CHS
6.8	g4560
3.3	m291
7.8	
	g4715_b
39.3	
	m247
4.6	g4028
3.8	APT
20.6	
	m435
8.2	
	g2368
6.3	m555
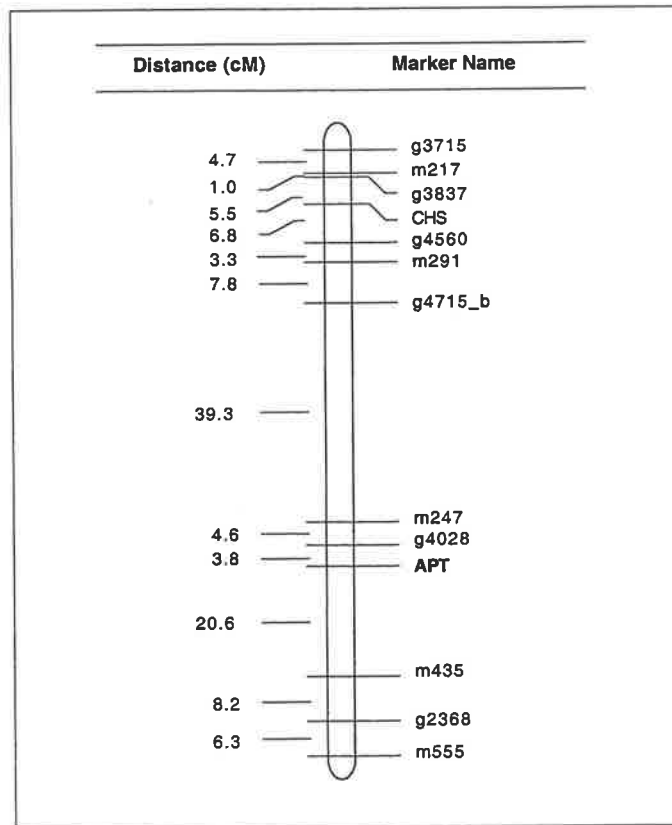
Fig. 4.4. Location of *APT* genes on chromosome 5 of *Arabidopsis* determined by Southern blot analysis of recombinant inbred lines.

4.3.2. Northern blot analysis of *APT* gene expression

Northern blots were used to study the expression of *APT1* and *APT2* genes. RNA was extracted from the roots of *Arabidopsis* plants that had either been continuously supplied with external Pi (+P) or deprived of Pi for 72 h (-P). Northern blots probed with *APT1* cDNA indicated that either both *APT1* and *APT2* or one of them were expressed in root tissues (Fig. 4.5). The level of expression in roots was considerably enhanced when plants were deprived of external Pi for 72 h. Expression was undetectable by Northern blot analysis with total RNA isolated from leaves, stems, flowers or siliques of plants either supplied or deprived of Pi for 72 h (data not shown). Further, the level of expression in roots was increased up to 2-fold when seedlings were deprived of N or K for 72 h compared with the 5- to 8-fold increases observed under P deficiency, indicating a degree of specificity for the response to P starvation (Fig. 4.6).

The expression of *APT1/2* in *pho1* and *pho2* roots showed a similar pattern to wild type plants with transcripts enhanced under P deficiency (Fig. 4.7). As found for wild-type plants, expression of *APT1/2* was undetectable in shoots, flowers or siliques of *pho2* mutants (data not shown).

4.3.3. Gene expression in relation to Pi uptake and internal Pi concentrations

Expression of the *APT1/2* genes was further studied in relation to Pi uptake and internal Pi concentrations in wild-type *Arabidopsis*. These were conducted under conditions where plants were i) running into P-deficiency; or ii) recovering from P-deficiency.

4.3.3.1. Plants running into P-deficiency

Arabidopsis plants were grown in full-strength nutrient solution for 23 d and then transferred to a -P solution for an additional 7-d growth. During the period of P deprivation, measurements of internal Pi concentrations, expression of *APT1/2* and Pi uptake were made at 0, 1, 2, 4, and 7 days after transferring to -P solution. Phosphate concentrations in both

+P -P
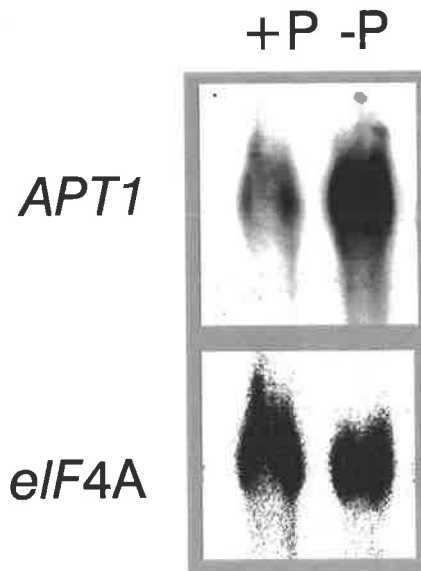
APT1

eIF4A

Fig. 4.5. Northern blot analysis of RNA (10 µg/lane) using the *APT1* cDNA as a probe
and *eIF*4A as a control. RNA was extracted form roots of wild-type *Arabidopsis* (30-d-
old) grown in full-strength nutrient solution with P (+P) and without P (-P) for the last
72 h of growth. The bands were quantified with a PhosphoImager system and the
increase in expression observed in the -P treatment relative to the +P treatment (5-fold)
was calculated after taking into account different loadings of RNA with the *eIF*4A
control

+ -P -N -K
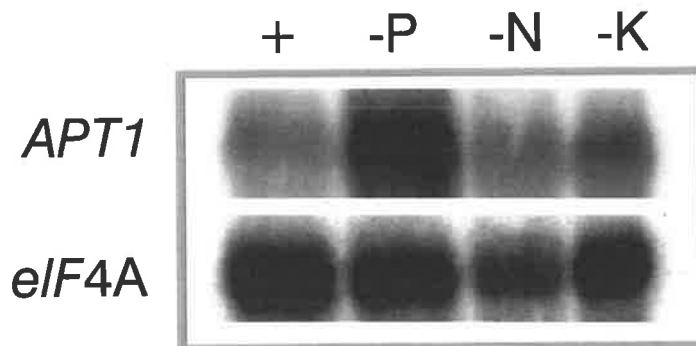
APT1

eIF4A

Fig. 4.6. Northern blot analysis of RNA (5 µg/lane) using the *APT1* cDNA as a probe
and *eIF*4A gene as a control. RNA was extracted from roots of wild-type *Arabidopsis*
(30-d-old) that were grown in hydroponic culture with all nutrients supplied (+),
without P (-P); without N (-N) and without K (-K) for the last 72 h of growth. The
bands were quantified with a PhosphoImager system and the increase in expression
observed in the minus treatments relative to the plus treatment (4.8-fold for -P, 2.3-fold
for -N and 1.8-fold for -K) was calculated after taking into account different loadings of
RNA with the *eIF*4A control.
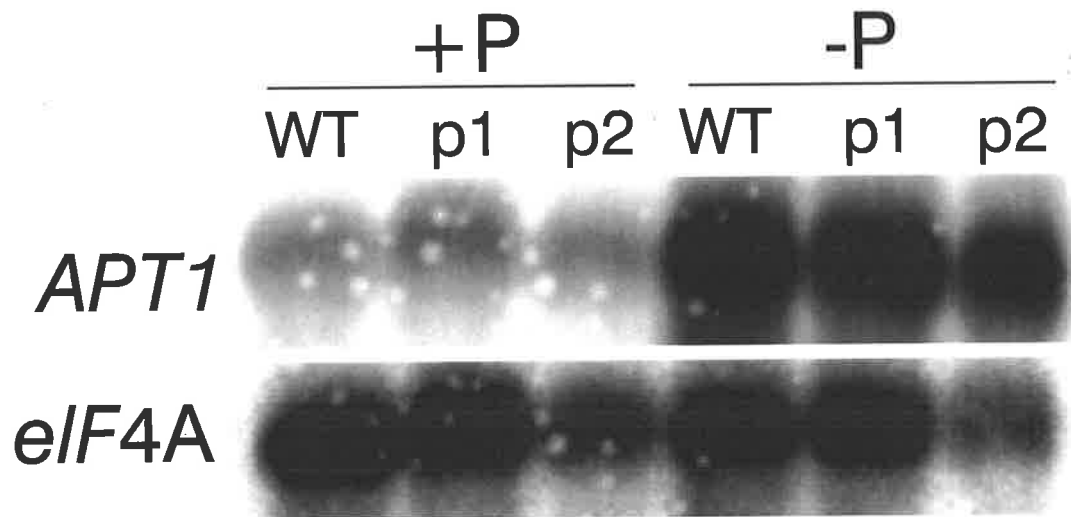
Fig. 4.7. Northern blot analysis of RNA (5 μg/lane) using the *APT1* cDNA as a probe and *elF*4A gene as a control. RNA was extracted from roots of wild type (WT) *Arabidopsis* plants, *pho1* mutants (p1) and *pho2* mutants (p2) (30-d-old) grown in hydroponic culture either continuously with Pi (+P) or transferred to Pi-free solution (-P) for the last 72 h of growth. The bands were quantified with a PhosphoImager system and the increase in expression observed in the -P treatments relative to the +P treatments (8.4-fold for wild type, 5.3-fold for *pho1* and 6.4-fold for *pho2*) was calculated after taking into account different loadings of RNA with the *elF*4A control.

shoots and roots decreased steadily throughout the period as expected, and after 7 d, the Pi concentrations in shoots and roots were reduced by 80% and 90% of the non-starved plants, respectively (Fig. 4.8a). Despite these substantial changes in Pi nutrition, only minor symptoms of P deficiency were detected. The relative expression of the *APT1/2* gene in plant roots increased during the period of P deprivation (Fig. 4.8b). The largest increase occurred between 2 and 4 d, and by 7 d, expression of *APT1/2* was about 5-fold higher than the expression in P-sufficient plants.
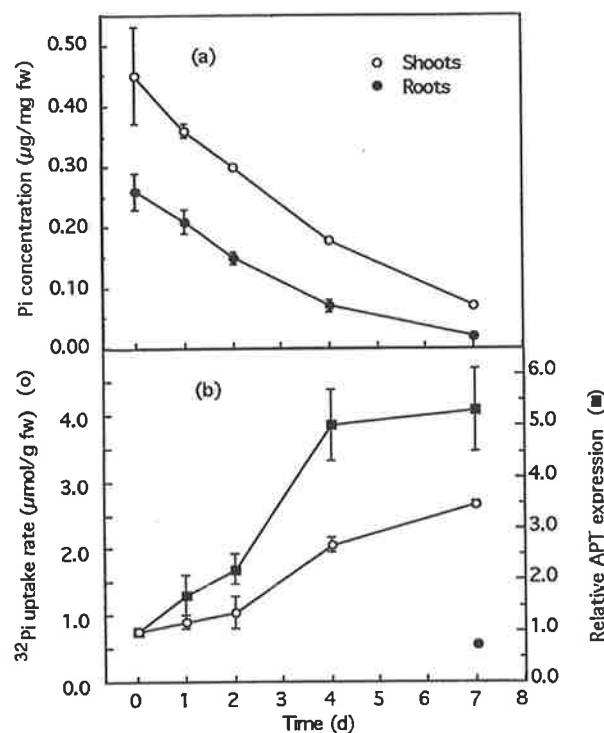
Fig. 4.8. Inorganic phosphate concentrations in shoots and roots (a) and ^{32}Pi uptake rate and relative expression of *APT1/2* in roots of *Arabidopsis* deprived of Pi (b). The error bars represent SE for the mean of three replicates when greater than the symbols. The filled circle in (b) represents the ^{32}Pi uptake rate of control plants which were supplied with Pi all the time. The relative expression of *APT1/2* was arbitrarily set at 1.0 for the control plants (+P) at day 0.

Pi uptake was estimated by two techniques: (i) accumulation of ^{32}Pi from a single external concentration of ^{32}Pi, and (ii) Pi-dependent changes in membrane potential difference (Em). When Pi moves across a membrane, it is co-transported with nH^+ (n>2, Sakano, 1990; Dunlop and Gardiner, 1993) and the membrane potential decreases due to the net movement of positive charge into the cells. Therefore, membrane depolarisation is closely related to Pi influx into cells. Figure 4.8b showed that the increase in ^{32}Pi uptake paralleled the changes in *ATP1/2* expression. Uptake was relatively unchanged for the first two days and then a large increase occurred between days 2 and 4. Unlike the ^{32}Pi experiments, where it was possible to estimate Pi uptake by plants well supplied with Pi, Pi-dependent changes of Em in the roots of P-sufficient plants were undetectable in these plants. However, when plants were deprived of Pi for 1 d or more, the addition of Pi caused the membrane to depolarise (become less negative) in a concentration-dependent manner (Fig. 4.9a). The Pi-dependent changes in membrane potential increased with an increase in Pi concentration to approximately 20 µM and showed no further increase with 50 µM Pi. The data were reasonably well described by the Michaelis-Menten equation with values for r^2 reaching 0.58, 0.92 and 0.78 for data from the 4, 6 and 7 days of P-starvation, respectively. The kinetic parameters of Km and Vmax were estimated from pooled data collected at each time point. The Vmax of Pi uptake increased from 2 mV after 1 d of Pi starvation to almost 30 mV after 7 d (Fig. 4.9b). By contrast, the estimated values for Km showed no significant changes, remaining at approximately 6 µM throughout the 7-d starvation period. The relatively large error bars associated with the earlier time points (1 and 2 d after P-deprivation) reflect greater proportionate errors in estimating the Km for low uptake rates.

4.3.3.2. Resupply of Pi to P-starved plants

When 33-d-old plants were deprived of Pi for 7 d and then resupplied with Pi, restoration of Pi concentrations in shoots and roots was rapid (Fig. 4.10a). After the first day of resupplying Pi, concentrations of Pi in the shoots increased to a value which is considered adequate for many plant species (Reuter and Robinson, 1986). In roots, the Pi concentration increased during the first day only and then remained unchanged (Fig. 4.10a).

APT1/2 expression decreased 80% within the first day of Pi resupply (Fig. 4.10b). The rate of ^{32}Pi uptake was reduced by about 30% after 1 d, and after 2 d, uptake declined to similar rates measured in the control plants that were fed Pi continuously (Fig. 4.10b).
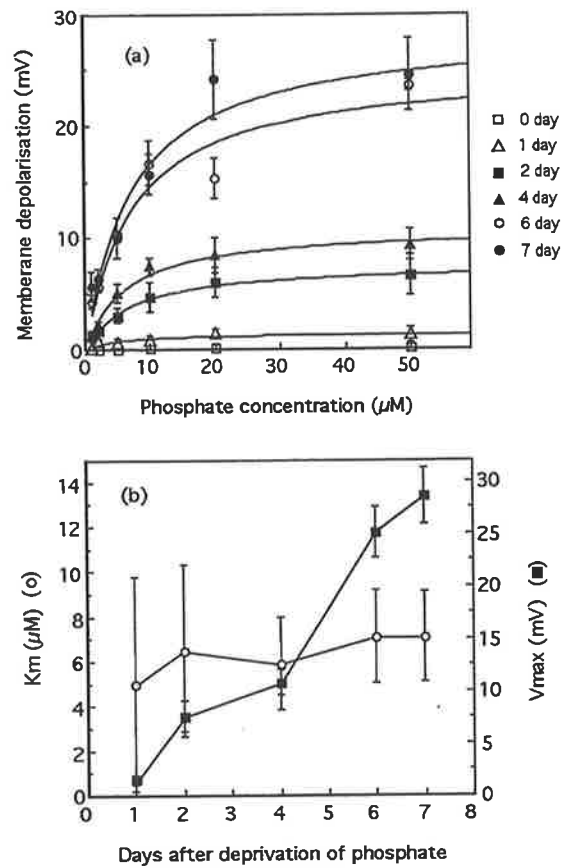
Fig. 4.9. Phosphate-dependent membrane depolarisation (a) and the resulting kinetic parameters Km and Vmax of Pi uptake by wild-type *Arabidopsis* roots deprived of Pi (b). The error bars represent SE of the mean of two replicates, and there were 3-6 measurements for each plant.
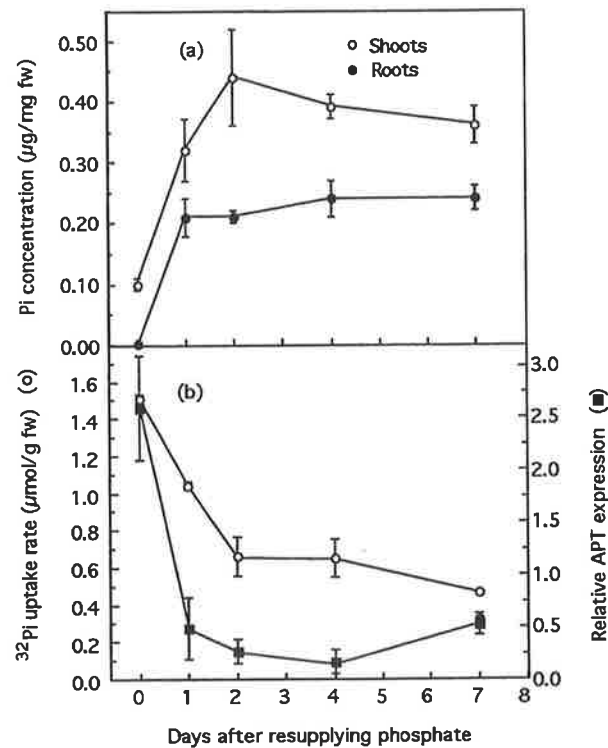
Fig. 4.10. Pi concentrations (a) and ^{32}Pi uptake rate and relative expression of *APT1/2* (b) in P-starved wild-type *Arabidopsis* when resupplied with Pi. The error bars represent SE for the mean of three replicates. The relative expression of *APT1/2* was arbitrarily set at 1.0 for the control plants (+P). The filled circle (overlapped by the filled square at day 7) in (b) represents the ^{32}Pi uptake rate of control plants which were supplied with Pi all the time.

Phosphate-dependent changes in Em were also monitored during the resupply of Pi to P-starved plants. Unlike the results with ^{32}Pi, which found Pi uptake had stabilised within 2 days, the electrophysiological estimates of Vmax of Pi uptake continued to decrease for up to 4 days (Fig. 4.11).

4.4. Discussion

4.4.1. *APT1/2* encode genes belonging to a Pi transporter family

Southern blot analysis showed that *APT1/2* belong to a small family of genes that are likely
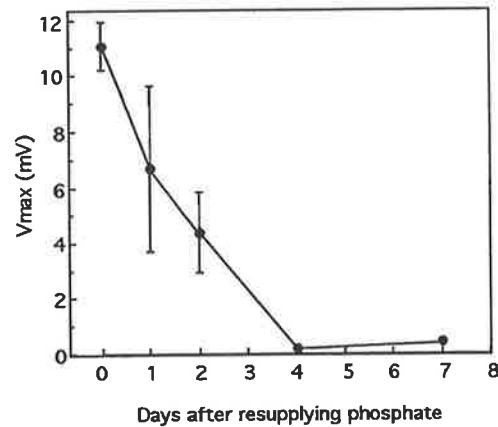
Fig.4.11. Vmax of Pi uptake obtained from membrane depolarisation measurements of starved wild-type *Arabidopsis* roots when resupplied with Pi. The error bars represent SE for the mean of two replicates with 3-6 measurements for each plant.

to be involved in Pi uptake or transport processes based on their sequence similarity to *PHO84* in yeast. At about the same time that the *APT1/2* genes were isolated, Muchhal et al. (1996) reported the cloning of cDNAs (*AtPT1* and *AtPT2*) from *Arabidopsis*. The coding region of *AtPT1* was identical to the coding region of *APT2* and was able to successfully complement the yeast *pho84* mutant. Another group have isolated a gene designated *PHT1*, which is also identical to *APT2*. *PHT1* was successfully overexpressed in tobacco-cultured cells and an increased Pi uptake with a Km of 3.3 μM was observed (Mitsukawa et al., 1997b). These results confirm that the *APT2* gene encodes a Pi transporter and based on its strong similarity to *APT1*, it is also likely that *APT1* encodes a Pi transporter.

4.4.2. *APT1/2* genes are regulated by Pi nutrition

The expression of the *APT1/2* genes are specifically regulated by P nutrition (Fig. 4.6). Northern blot analysis showed that they are primarily expressed in roots indicating a probable role in Pi acquisition by roots. Under P deficiency, expression of the genes in roots increased about 5-fold suggesting that the synthesis of Pi transporters increases. This

is consistent with the Pi uptake kinetic studies that Vmax increased under the same conditions (Fig. 3.4, Chapter 3).

A more detailed study on the relationships between *APT1/2* genes expression, Pi uptake rates and internal Pi concentrations showed that as the plants moved into P-deficiency, increased expression of *APT1/2* initially responded slowly (Fig. 4.8b). By contrast, the expression of the genes in P-starved plants was decreased relatively quickly when resupplied with Pi (Fig. 4.10). This might be because the expression does not increase until Pi concentrations within the plant fall below a critical value or, that expression of the genes depends on a specific Pi pool, rather than the total amount of Pi. Such a pool may be slow to deplete when plants progress into deficiency but is quickly replenished during resupply of Pi. The higher Pi uptake rate of starved plants resupplied with Pi after 1 d than that of the control plants, regardless of expression of the transporter gene being reduced to a basal level (Fig. 10b), is consistent with the observation made by Clarkson et al. (1992). Using inhibitors of protein synthesis, they concluded that, unlike sulphate transporters which turned over rapidly with a half-time of about 2.5 h, Pi transporters are likely to be turned over more slowly. This will mean that the number of transporters will respond more slowly to Pi resupply than mRNA levels.

Although the expression of the *APT1/2* genes was correlated with Pi uptake rates and both genes were induced by P deficiency, it is unknown where these genes are expressed in root tissues and whether the proteins that they encode are in fact responsible for Pi uptake into the root cells. For example, *APT1* and *APT2* may be located in the stellar cells or in the tonoplast of root cells, neither of these is responsible for Pi uptake directly from the external medium by plant roots but may indicate a role in transport of Pi within the plant. Further work using *in situ* hydridisation with *APT1/2* and immunochemical localisation with their antibodies will provide evidence regarding the functions of these genes. *In situ* hybridisation using a *LePT1* probe for the Pi transporter mRNA from tomato, which shows homology with the *APT1/2* genes, demonstrated that expression of this gene occurs in epidermal cells of tomato roots under Pi starvation (Liu et al., 1998). More recently, immunochemical localisation using antibodies raised against the protein encoded by *LePT1*,

has shown that the LePT1 is located on the plasma membrane of these epidermal cells and that the amount of LePT1 is increased by Pi starvation (Muchhal and Raghothama, 1998). This result adds additional weight to the suggestion that this protein is involved primarily in Pi uptake from the external medium.

4.4.3. Specific expression of *APT1* and *APT2*

Since it was not possible to differentiate between the expression of *APT1* and *APT2* in Northern blots using full-length cDNA probes, Dr. Frank Smith's group also used the 5' untranslated regions of both genes as probes. The Northern blot results confirmed the coordinated induction of both *APT1* and *APT2* genes in -P roots (Smith et al., 1997). Furthermore, they used RT-PCR to look for low levels of expression in other tissues. Different primer pairs were used to amplify *APT1* and *APT2* reverse transcription products from leaves, roots, flowers and siliques of +P and -P *Arabidopsis*. High yield of amplification products by both primers were obtained in -P roots and low yield in +P roots. There were no products from RNA derived from other organs except for a low level of product obtained from -P leaves with *APT2*-specific primers. The identical coding regions of *APT1* and *APT2* suggest similar structure and functions for these proteins. The completely different sequences of the untranslated and promotor regions may contain specific information which controls the expression of the genes. This may explain detectable expression of *APT2* in -P leaves while expression of *APT1* was not detected under the same conditions.

4.4.4. Relationship of the *APT1* and *APT2* genes to the *pho1* and *pho2* mutants

It is possible that *pho1* or *pho2* mutants result from mutations in either of the Pi transporter genes described in this chapter or in one of the other recently cloned Pi transporter genes. However, *pho1* was mapped to chromosome 3 and *pho2* to chromosome 2 (Delhaize and Randall, 1995), while *APT1* and *APT2* were mapped to chromosome 5 (Fig. 4.4). This indicates that mutations in *APT1* and *APT2* are not responsible for either *pho1* or *pho2*.

Furthermore, a similar expression pattern of the genes in roots of the mutant and wild-type plants suggests that *PHO1* and *PHO2* (the wild-type genes) are not involved in regulating the transcription of *APT1* and *APT2*. In addition, the results indicate *APT1/2* transcription is responding to P status in roots, rather than that in leaves, because Pi concentrations in roots of the mutants are similar to those of wild type while the Pi concentrations in shoots are either excessive (*pho2*) or deficient (*pho1*). If the expression of *APT1* and *APT2* genes in roots were being controlled by Pi in shoots, then I would have expected expression to be altered in the mutants.

4.4.5. Phosphate uptake

Phosphate uptake was examined by Pi-induced membrane depolarisation as well as by ^{32}Pi uptake. The results obtained with these two techniques were largely similar but there were some differences: (i) restoration of Pi in P-starved plants decreased Pi-induced membrane depolarisation to the control level by day 4 (Fig. 4.11), while ^{32}Pi uptake declined to control levels by day 2 (Fig. 4.10b); and (ii) uptake was measurable in P-fed plants with ^{32}Pi but membrane depolarisation could not be detected (Figs. 4.8b and 4.9a). There are several possible explanations for these differences. First, membrane depolarisation is a transient process occurring in an electrical contiguous region of roots and could be obtained in less than 1 min. Thus it measures a rapid Pi influx into root cells. By contrast, ^{32}Pi uptake was conducted over 1 h and represents an average uptake by the plants. It involves different cell types including shoot cells, because within 1 h some of the ^{32}Pi taken up by the roots is transported to shoots. The existence of different kinds of Pi transporters in the plasma membrane, for example, a charge-balanced $H^+/H_2PO_4^-$ co-transport such as the neutral transport of sulfate (Borstlap and Schuurmans, 1998) is another possible explanation for the different results obtained with the two techniques. This Pi transporter may operate all the time regardless of P nutrition. Therefore, Pi uptake through these transporters would not be detected by electrophysiological methods because there would be no net movement of charge across the plasma membrane. Different mechanisms responsible for Pi uptake by P-sufficient and P-deficient plants was also suggested by Dunlop and

Gardiner (1993). Since Pi was assumed to be co-transported with protons (Ullrich-Eberius et al., 1984; Sakano, 1990), Dunlop and Gardiner studied the effects of a proton translocating ATPase inhibitor on proton efflux, Pi uptake rate and membrane potential in P-deficient and P-sufficient plants. They found that the inhibitor only reduced the Pi uptake by P-deficient plants and P-induced membrane depolarisations could only be detected in P-deficient plants. These results suggest that there are qualitative differences in the mechanisms of Pi uptake under different conditions of P nutrition.

Kinetics of Pi uptake with the Vmax increasing and the Km remaining constant obtained by electrophysiology studies (Fig 4.9) is consistent with other studies using a range of plant species (Drew et al., 1984; Ullrich-Eberius et al., 1984; Furihata et al., 1992; Dunlop et al., 1997). A Km of 12 µM was reported by Dunlop et al. (1997) using used a ^{32}Pi uptake method. In the experiments presented here, a Km of about 6 µM was obtained (Fig. 4.9). Possible reasons for the differences in Km between the two studies could be due to the different methods applied as discussed above. In addition, Dunlop et al. (1997) used 3-d-old plants completely immersed in nutrient solution for uptake studies, whereas 23- to 30-d-old hydroponically cultured plants with only roots immersed in nutrient solution were used for the present study. Plants of different ages have shown different Pi uptake rates and kinetic parameters in several studies (Bar-Josef, 1973; Clarkson and Scattergood, 1982; Lefebvre and Glass, 1982).

4.5 Summary

In this chapter, two recently isolated genes *APT1* and *APT2* which are homologous to the yeast Pi transporter gene *PHO84*, were studied by Southern and Northern blot analysis. Southern blot analysis suggests there are multiple copies of homologous genes present in *Arabidopsis* genome. Both genes were mapped to chromosome 5 indicating that they are not responsible for either *PHO1* or *PHO2*. The transcripts of *APT1/APT2* genes were primarily found in root tissues and were strongly enhanced by P starvation. The expression of the genes correlated well with Pi uptake capacities and was iversely correlated with internal Pi

concentrations in plants. The results provide evidence that the *APT1/APT2* genes are involved in a Pi uptake system which is regulated by P starvation.

Chapter 5

Mapping of PHO2 on chromosome 2

5.1. Introduction

In the previous chapter, I concluded that the *pho2* mutant was not mutated in either *APT1* or *APT2* and also did not appear to regulate these genes at a transcriptional level. However, *PHO2* could be mutated in a shoot specific Pi transporter gene or in a gene involved in regulation of Pi uptake. Cloning *PHO2* and other Pi transporter genes will help us to gain a better understanding of Pi uptake and its regulation in higher plants.

General strategies to clone genes from higher plants include: 1) cross-hybridisation with genes from other species to identify a gene encoding a protein of similar function; 2) screening libraries with cDNA probes derived from tissue- or treatment-specific RNA; 3) screening of cDNA expression libraries with antibodies or oligonucleotide probes derived from a protein sequence; 4) DNA-tagging approaches using Ti plasmid DNA or transposons, and 5) map-based positional cloning. The first three strategies are dependent on considerable knowledge of the DNA sequence, protein sequence or product and expression pattern of the gene to be cloned. DNA insertion refers to methods of identifying genes by a mutagenic process involving insertion of foreign, but well-characterised DNA. Since the sequence of the DNA tag is known, there are a number of procedures that can be used to clone the sequences that are adjacent to the DNA tag. In these cases, mutants generated by DNA insertion must first be identified. Positional cloning, where the gene is cloned based on its chromosomal map position, is applicable for isolating any mutated gene whose phenotype is known but the product is not. For *Arabidopsis*, since it has the smallest genome in higher plants (7×10^7 bp) and remarkably low content of repetitive DNA (Pruitt and Meyerowitz, 1986), cloning approaches such as DNA tagging and map-based cloning are relatively efficient procedures.

The *pho2* mutant described in previous chapters was generated from the Columbia ecotype

of *Arabidopsis* using ethyl methylsulfonate (Delhaize and Randall, 1995) and a tagged mutant has not yet been identified. The phenotype of the mutant is known but the nature of the gene product and its action are not. Therefore, in this case, map-based positional cloning is the strategy of choice.

The first step in map-based cloning is to identify the chromosomal location of the mutated gene by analysing its co-segregation with other known morphological or genetic markers. A large number of mutants with morphological characters have been generated and mapped by classical linkage analysis (Koornneef, 1987). These mutations are distributed over the five chromosomes of *Arabidopsis* and can be conveniently used as markers to determine the relative position of other genes. Second, DNA markers residing near the locus of interest need to be identified. This high resolution mapping can be achieved by mapping the mutation relative to appropriate RFLP (restriction fragment length polymorphism), PCR-based CAPS (cleaved amplified polymorphic sequence), RAPD (random amplified polymorphic DNA) or SSLP (simple sequence length polymorphism) markers to bridge the intervening gap between the nearest marker and the target locus. Usually, a line containing the mutant gene to be mapped and flanking markers is crossed to a different ecotype with wild-type alleles. Recombinants between the mutant gene and the flanking markers in an F_2 population are selected, and DNA from these recombinants is further analysed with various markers to determine the precise position of the mutation. Classic genetic linkage and RFLP linkage maps of the *Arabidopsis* genome with hundreds of markers (Koornneef, 1987; Chang et al., 1988; Nam et al., 1989) have been constructed. The use of RAPDs, CAPSs and SSLPs integrated with new markers contribute to high density maps that are available for this type of positional cloning (Hauge et al., 1993; Konieczny and Ausebel, 1993; Bell and Ecker, 1994).

To facilitate cloning of the gene, some laboratories have constructed libraries of the *Arabidopsis* genome in yeast artificial chromosome (YAC) vectors (Ward and Jen, 1990; Grill and Somerville, 1991; Creusot et al., 1995). These YAC vectors contain all of the sequences necessary for stable maintenance in the yeast *Saccharomyces cerevisiae* and accept *Arabidopsis* genomic DNA inserts of several hundred or even a thousand kb. Once

the two nearest flanking markers for the region of interest are determined, chromosome walking is then initiated by using these flanking markers to search a YAC library for clones that hybridise to these probes. The ends of identified YACs are cloned and used in recombinants to determine the distance and orientation of the walk. Also, they are used for probing further YACs with overlapping DNA fragments. This is repeated until a region spanning the target gene is achieved. To further define the location of the target gene, libraries of BACs (bacterial artificial chromosomes) and cosmid clones containing smaller *Arabidopsis* genomic DNA fragments can be used (Choi et al., 1995). The physical maps, where the overlapped YACs are used to link up all of the RFLP markers on the five *Arabidopsis* chromosomes are being constructed. This has enabled the progress of cloning to be more direct and less time-consuming.

The final step is to subclone DNA from a clone that contains the target gene into plant transformation vectors and to complement the mutant phenotype. *Arabidopsis* can be easily transformed by an *Agrobacterium* root method (Valvekens et al., 1988) or vacuum infiltration transformation procedures (Bechtold et al., 1993).

There are a number of *Arabidopsis* genes that have been isolated by using the map-based cloning approach such as *FAD3, ABI3, LEAFY,* and *CO* (Arondel et al., 1992; Giraudat et al., 1992; Weigel et al., 1992; Putterill et al., 1993), and it is becoming a routine method when numerous polymorphisms are available in the region of interest. However, some obstacles remain when applying this method. The problem could be due to repetitive DNA, gaps in various libraries, low density of polymorphisms in the region being mapped resulting in a lack of appropriate flanking markers, and variations between genetic and physical distances. In the near future, completion of YAC, BAC, and cosmid contigs and their integration with genetic markers will contribute to a genetic map with higher resolution. In addition, the *Arabidopsis* genome is being sequenced. The project, known as the *Arabidopsis* genome initiative (AGI), aims to obtain an accurate sequence of the *Arabidopsis* genome. The entire *Arabidopsis* genome is expected to be completely sequenced by the year 2004 and will greatly facilitate the cloning of genes by the map-based approach.

The *pho2* mutant has been crossed to the tester lines that carry recessive phenotypic markers (including *ap1, gl2, ch1, cp2, as1, hy2, gl1, bp1, ap2, yi1* and *ttg1*) for the various chromosomes (Delhaize and Randall, 1995). In the F_2 generation, seedlings with the chromosome markers were analysed for Pi concentration and *pho2* was shown to be linked to *as1* on chromosome 2 (Delhaize and Randall, 1995). This was further confirmed with the CAPS marker *m429* which is also located on the lower arm of chromosome 2. A physical map of chromosome 2 of *Arabidopsis* consisting of YAC contigs that cover 90 % of the chromosome has been established (Zachgo et al., 1996). In addition, as part of the *Arabidopsis* Genome Initiative mentioned above, about 7 Mb on chromosome 2 has been sequenced (The Institute for Genomic Research, Rockville, MD, USA. Website: http://www.tigr.org/tdb/atgenome/at_bacs.html). These attributes together provide a sound basis for the cloning of *PHO2*. In this chapter, the fine-mapping of *PHO2* is described as a precursor to cloning the gene.

5.2. Materials and methods

5.2.1. Plant growth and screening for the *pho2* phenotype

Plants grown in composted soil as described in Section 4.2.1 were used to undertake genetic crosses and to screen for recombinants. Under some conditions, the *pho2* phenotype is not visually distinctive, and in order to identify the *pho2* mutants, a piece of leaf from each selected plant (about 6-10 mg) was taken for Pi assay. Leaves were ground with a drop of 5 M H_2SO_4 in a microfuge tube and 1 ml of distilled water was added. The mixture was vortexed, centrifuged (10,000 × *g*) for 5 min and the supernatant was was used for Pi assay with the malachite green reagent (Chapter 2.2.2).

5.2.2. Genetic crosses

Flowers of the female parent were emasculated with forceps to prevent self-fertilisation and pollinated by touching the stigma with anthers from the male parental plant. After fertilisation, the ovary elongates and develops into a silique. Seeds were collected from

single siliquae and analysed in further experiments.

5.2.3. Plant DNA isolation and RFLP analysis

Genomic DNA from shoots was extracted and digested with restriction enzymes, fractionated on agarose gel and transferred to nylon filters as described in Chapter 4.2.2. The filter was probed with genomic clones (obtained from the Arabidopsis Biological Resource Center, Ohio, USA) and cloned YAC ends from yUP libraries (University of Pennsylvania, Philadelphia, USA).

About 100 ng primers of each CAPS markers (Table. 5.1) were used for PCR. Purified genomic DNA was diluted by 10- or 100-fold. The conditions for the PCR consisted of 35 cycles of the following sequence; 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 min. After PCR, the DNA products were digested with the restriction enzymes *Mse*I for the *cop1* marker or *Dde*I for the *TEn5* marker and polymorphisms were scored on a 2% agarose gel.

When mapping with SSLP marker, about 100 ng of the primers and 100-fold diluted plant genomic DNA were used for PCR. The conditions for the PCR consisted of 40 cycles of the following sequence; 94 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. The products were run on a 4% agarose gel.

Table. 5.1 Polymorphisms and primer sequences of the RFLP markers

		Fragment size, Enzymes: ecotypes, cuts (size of products in kb)	primers
CAPS marker	cop1	0.9 kb, MseI: Col, 3 (0.4, 0.29, 0.12, 0.075); Ler, 3 (0.38, 0.325, 0.12, 0.075)	5'-CCACTCAGCGCATCCTTC-3' 5'-GCTCGGCATGTGTCAAAA-3'
CAPS marker	TEn5	1.56 kb, DdeI: Col, 5 (0.742, 0.293, 0.22, 0.207, 0.066, 0.031); Ler, 6 (0.742, 0.293, 0.207, 0.13, 0.09, 0.066, 0.031)	5'-GAGATATTGATTGGTGTGAC-3' 5'-CGAAGTTCTTGAGCAACTAA-3'
SSLP marker	nga361	Col (114 bp) L.*er* (120 bp)	5'-AAAGAGATGAGAATTTGGAC-3' 5'-ACATATCAATATATTAAAGTAGC-3'

End sequencing of BAC clone T14G11was used to find a polymorphism between Col and L.*er* and subsequently used to score the recombinants.

5.3. Results

5.3.1. Isolation of a *pho2/as1* double mutant

To facilitate the mapping of *PHO2*, a double *pho2/as1* mutant in the Col genetic background was constructed to enable easier selection of recombinants between *pho2* and *as1* (see Koncz et al., 1992 for a discussion of the use of double mutants). The *pho2* mutant was crossed to the chromosome 2 tester line that contains the recessive mutations *er1*, *as1* and *cer8*. Seed was collected and grown to generate seeds for the F2 generation. From this population, a plant with both *as1* (asymmetric, lobed rosette leaves, map position 73.4 cM, Website: http://www.aims.menu/search.html/) and *pho2* mutations was identified (Fig. 5.1). This double mutant, in addition to the *pho2* and *as1* phenotypes, showed phenotypes for *ER* (Columbia type flowers and siliqua, map position 56.5 cM) and *cer8* (bright green stems and siliques, map position 86.8 cM, Website: http://www.aims.menu/search.html/). These seedlings were allowed to self-fertilise and the seeds were collected to be grown for the next generation. All progeny showed *ER*, *pho2*, *as1* and *cer8* phenotypes which confirmed that these loci were homozygous. On the basis of these results, *PHO2* was likely to be located between *ER* and *AS1* (Fig. 5.2). Mapping of *PHO2* was conducted from both the *ER* side and the *AS1* side.

pho2mutant   chromosome 2 tester
                                              line

ER   pho2 AS1 CER   er   PHO2 as1   cer
☐   ▉
☐   X   ▉
ER   pho2 AS1 CER   er   PHO2 as1   cer

                    ER   pho2 AS1 CER
          F1   ☐
                    ▉
                    er   PHO2 as1 cer

                          ⊗

                    ER   pho2   as1 cer
          F2   ☐▉   Recombinant
                    ☐▉
                    ER   pho2   as1 cer
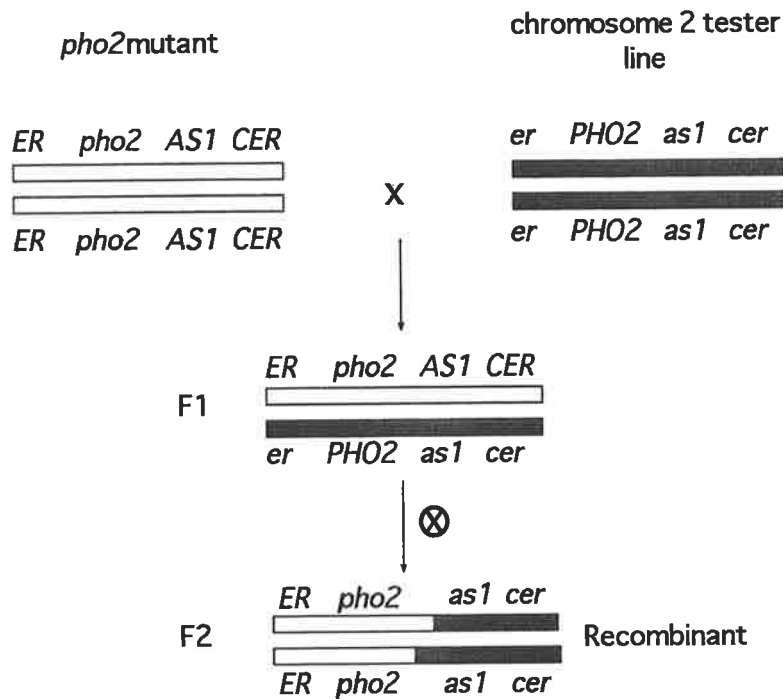
Fig. 5.1. Schematic diagram showing how a *pho2/as1* double mutant was generated from crossing the *pho2* mutant with chromosome 2 tester line. The positions reflect the likely location of *pho2* relative to other markers. Typical Mendelian segregation of 1:2:1 genotypes and other possible recombination genotypes in the population of F_2 are not shown in this figure. The chromosome 2 tester line is drawn as shaded bars for clarity. Both parental genotypes possess the Col genetic background in the region of interest.

N

ER ⊢— 56.5 cM

PHO2

AS1 ⊢— 73.4 cM

CER 8 ⊢— 86.8 cM

S
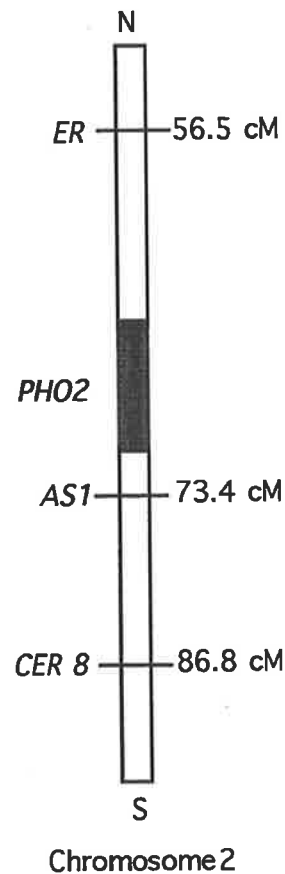
Chromosome 2

Fig. 5.2. A simplified diagram of *Arabidopsis* chromosome 2 showing the position of *PHO2* relative to other morphological and molecular markers (based on http://aims.menu/search.html/). The shaded region indicates the possible location of *PHO2*. North is proximal to the centromere.

5.3.2 Mapping of *PHO2* from the *as1* side

The *pho2/as1* double mutant was crossed to a wild-type L.*er* seedling and recombinants between *PHO2* and *AS1* were identified in the F₂ generation. The plants of the F₂ generation showing an *as1* phenotype were assayed for Pi concentration and those with Pi concentration in the range of wild-type seedlings were identified as *PHO2/as1* recombinants (Fig. 5.3). Genomic DNA was then extracted from each recombinant and at the same time, a few silique were kept to harvest seeds. If necessary, a bulk harvest of at least 16 plants in

Fig. 5.3. Schematic diagram showing genotypes resulting from a cross between Col background *pho2/as1* double mutant and wild-type L. *er*. The F_1 plants were allowed to self-fertilise. The F_2 generation would mainly be of the types shown in the first row of F_2 in an approximate 1:2:1 ratio. However, recombination between *PHO2* and *AS1* would be expected as shown in the second row of the F_2 population. To facilitate identification of recombinants, only plants with the *as1* phenotype were selected in the F_2 population and assayed for Pi. (The *pho2* phenotype is difficult to identify visually and selection of *pho2/AS1* recombinants would have required that more plants be assayed for Pi).

the F3 generation was used to extract additional genomic DNA. A more accurate position of

PHO2 was achieved by using RFLP markers in the region around the estimated location of

PHO2. An RFLP closer to the *PHO2* locus would show more heterozygous Col/ L.*er*

patterns and fewer homozygous Col patterns than an RFLP further away from *PHO2* as

shown in Fig. 5.4.
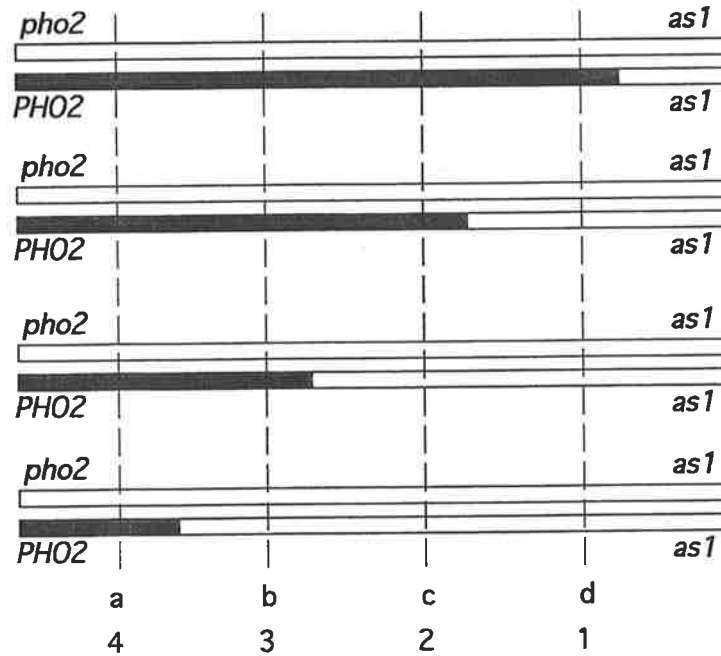
Fig. 5.4. Schematic diagram showing *PHO2/as1* recombinants scored with different probes a, b, c and d. The numbers at the bottom indicate the number of heterozygotes scored by the corresponding probes. The probe closer to the *PHO2* locus, for example, probe "a" will score more heterozygotes than the probes closer to *as* locus, "c" and "d". Recombinants with cross-over between *PHO2* and *AS1* on both chromosomes (as shown in Fig. 5.2) could sometimes give homozygous L.*er* patterns. The shaded bars represent L.*er* genetic background and the blank bars represent Col genetic background.

The genetic marker *m323* between *ER* and *AS1* was first used to screen a YAC library, and

clone yUP9D3 was identified. The left end of yUP9D3 was subcloned and used to identify

two other YAC clones yUP11D2 and yUP11A7. The right end of yUP11D2 (referred to as

11D2R) and the left end of yUP11A7 (referred to as 11A7L) were subcloned. These two

YAC-end clones (kindly provided by Mr. M. Luo, CSIRO Division of Plant Industry),

whose genetic position was identified as being between *m323* and *AS1* (personal communication with Mr. M. Luo), were used as probes to score the *PHO2/as1* recombinants. Polymorphisms for parental genomic DNAs probed with both YAC ends were found with a *Hind*III digestion and were used to score 14 *PHO2/as1* recombinants. Eleven of the recombinants were heterozygous and three were homozygous for the Col genotype when probed with 11D2R (Fig. 5.5). The same recombinants, when probed with 11A7L, showed four homozygous Col patterns (Fig. 5.5), indicating that 11D2R was closer to the *PHO2* locus (See Figure 5.6). Another genomic probe *mi277* (mapping position 72.2 cM, Website: http://weeds.mgh. harvard.edu/goodman/c2_d.html) was used to test the same 14 recombinants (digested with *Dra*I) resulting in only one recombinant with a homozygous Col pattern. Analysis of 12 additional recombinants with *mi277* all showed a heterozygous pattern. Assuming a random, but statistically even distribution of crossover events on chromosome 2 between *PHO2* and *AS1*, it indicates that *mi277* was close to, and to the south of the *PHO2* locus. The only recombinant with a homozygous Col pattern was further tested with the sequencing polymorphism marker T14G11 (which is north of *mi277*) and it still showed a homozygous Col. pattern. The CAPS marker *TEn5* (mapping position 69.7 cM, Website: http://weeds.mgh.harvard.edu/goodman/ c2_d. html), which is north of both *mi277* and T14G11, was then used to test this homozygous Col recombinant and yielded a Col/L.*er* heterozygous pattern. From these data, the relative positions of the probes and the *PHO2* locus are shown in Fig. 5.6.

5.3.3 Mapping of *PHO2* from the *er side*

Plants with recombination occurring between *ER* and *PHO2* were also selected from the F$_2$ generation of the *pho2/as1* double mutant by L.*er* cross. Recombinants with *er/pho2* phenotype were kept for RFLP analysis to determine the closest marker to *PHO2* from the *ER* side (Fig. 5.7, Type II plants). Flowers and siliquae of L.*er* (*er*) and Col (*ER*) are visually different (Fig. 5.8), and the *er* phenotype can be easily selected. However, as required for mapping from the *AS1* side, these plants with *er* phenotype needed to be analysed for their Pi concentration to confirm the *pho2* phenotype.
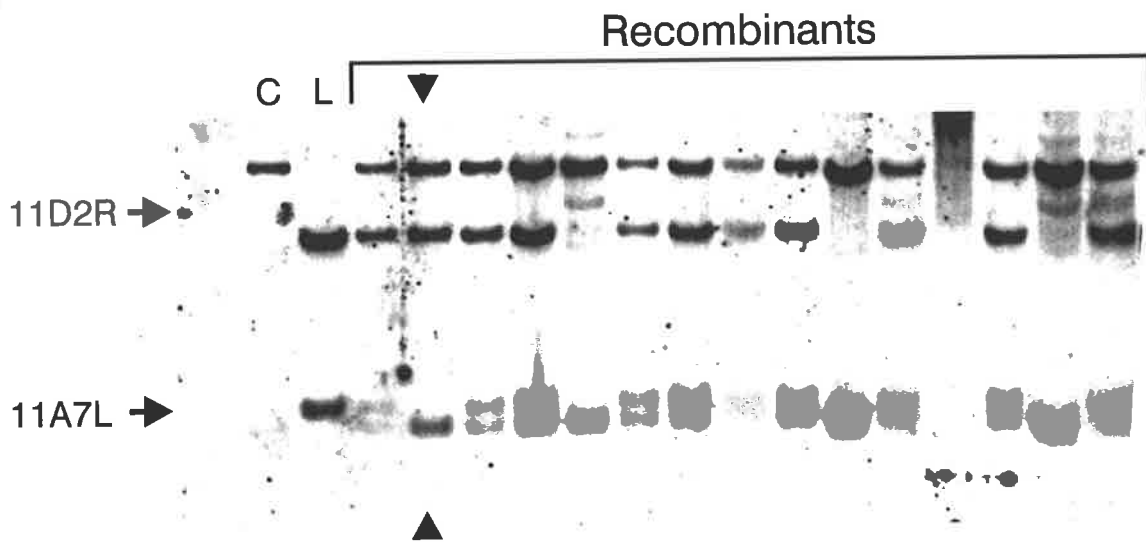
Fig. 5.5. Examples of *PHO2/as1* recombinants whose DNA were restricted with *Hind*III and hybridised with probes prepared from YAC ends 11D2R and 11A7L. The RFLPs defined by *Hind*III digestion are shown to be derived from the parental lines Columbia ("C") and Landsberg ("L"). Note that the arrows pointing to the hybridisation pattern of the recombinant is Columbia homozygous type when probed with 11A7L but changes to Columbia and Landsberg heterozygous type when probed with 11D2R.

chromosome 2

ER   TEn5   T14G11 mi277   m323   AS1

(0/1)   (1/1) (1/26)

yUP 9D3   9D3L

11D2R   yUP 11D2

North   (3/14)   11A7L   yUP 11A7

YACs

(4/14)
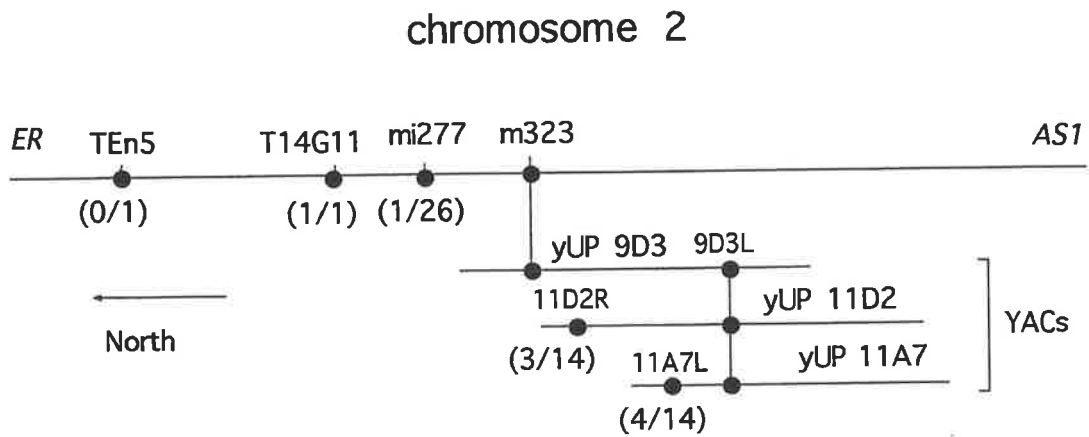
Fig. 5.6. Schematic diagram showing relative positions of the genes and probes used for screening *PHO2/as1* recombinants. The distances in the graph are not shown to scale. The left end of the YAC, refers to the DNA fragment adjacent to the ampicillin selection marker on the YAC; The right end of the YACs, refers to the DNA fragment cloned by homologous recombination in yeast followed by bacterial rescue with the pLUS vector. The left end of yUP11A7 (11A7L) and the right end of yUP11D2 (11D2R) were used as probes to score the *PHO2/as1* recombinants. Fractions in the brackets indicate the number of recombinants with a homozygous Col pattern from the total number of recombinants used for RFLP analysis. For markers T14G11 and *TEn5*, only the recombinant that showed homozygous Col pattern at *mi277* was tested. The direction of the arrow (North) is proximal to the centromere.
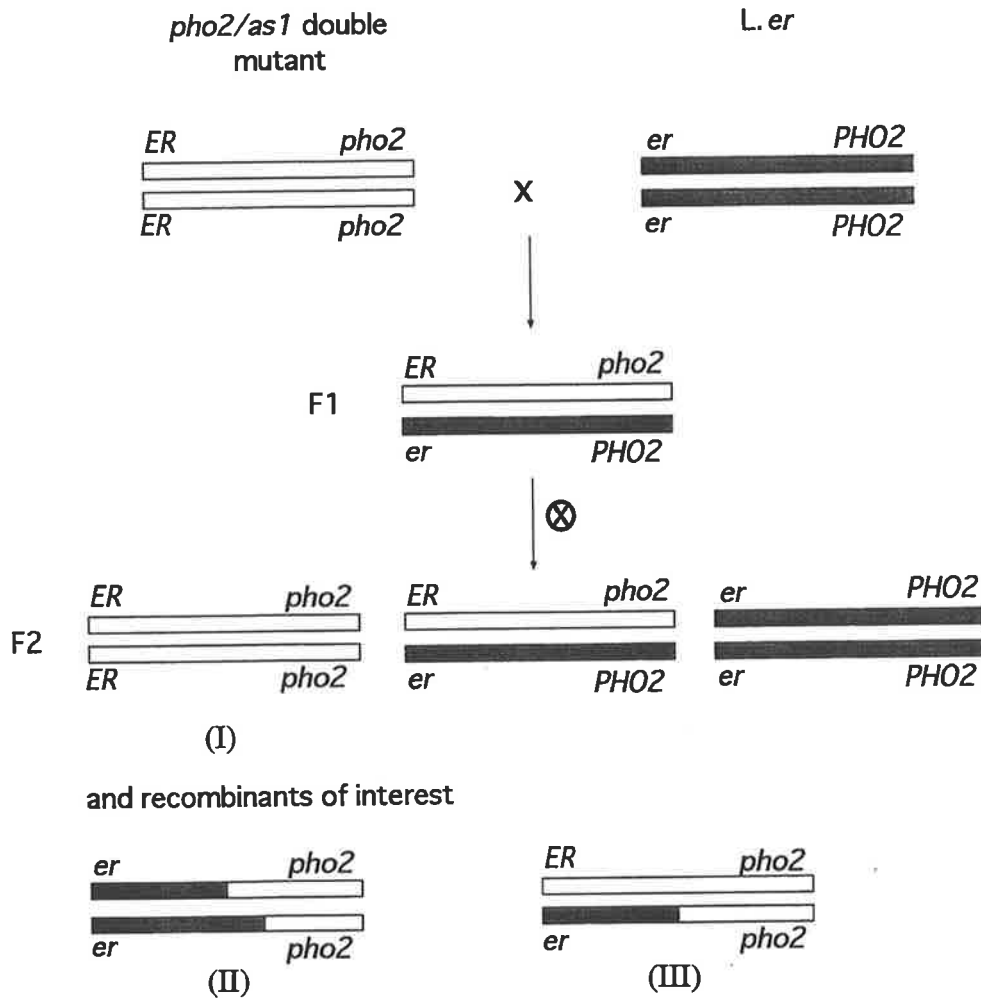
Fig. 5.7. Schematic diagram showing genotypes resulting from a cross between *pho2/as1* double mutant and a wild-type L. *er* plant and allowing self-fertilisation of F$_1$ plants. The F$_2$ generation would mainly be of the types shown in the first row of F$_2$ in a 1:2:1 ratio. However, recombination between *ER* and *PHO2* would be expected as shown in the second row of F$_2$ plants and were used in this study (Type II and III). Other possible recombinants are not shown.

This selection strategy, however, is less efficient than selecting *PHO2/as1* recombinants (the double recessive approach) because only the double exchange recombinants can be selected. Although the double recessive mutant of *er/pho2* in a Col phenotype could not be generated due to *er* being derived from the L.*er* genetic background, I kept all seedlings with an *ER/pho2* phenotype in the F$_2$ generation to increase the population of plants with
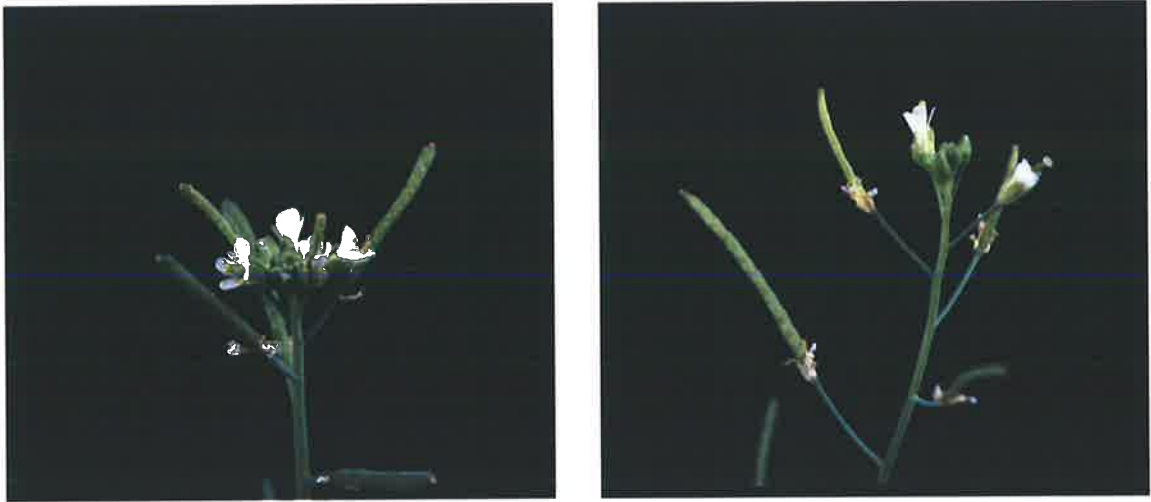
Fig. 5.8. Flowers and siliquae phenotypes of Landsberg *erecta* (L.*er*, left) and Columbia (Col, right) of *Arabidopsis*.

recombinants between *pho2* and *er*. These *ER/pho2* seedlings would consist of plants homozygous for *ER* and therefore not recombinants (Type I, Fig. 5.7) and plants heterozygous at the *ER* locus (Fig. 5.7. Type III plants) with the same phenotype, but in this case are recombinants and can be analysed with molecular markers. To select the Type III plants from Type I, the seeds of each plant (*ER/pho2* phenotype) were collected and allowed to self-fertilise to produce the F3 generation. In the F3 population, plants showing segregation of *ER* and *er* (3:1, parents are Type III plants in Fig. 5.7) were bulk harvested, and genomic DNA was extracted. Available molecular markers between *er* and *as1* were used to score these recombinants. In this case, a probe which is closer to the *PHO2* gene from the *ER* side should score more homozygous Col patterns and fewer Col/L.*er* heterozygous patterns (Fig. 5.9). Taking this approach, I identified 18 *ER/pho2* recombinants. These plants were first analysed with the SSLP marker *nga361* (mapping
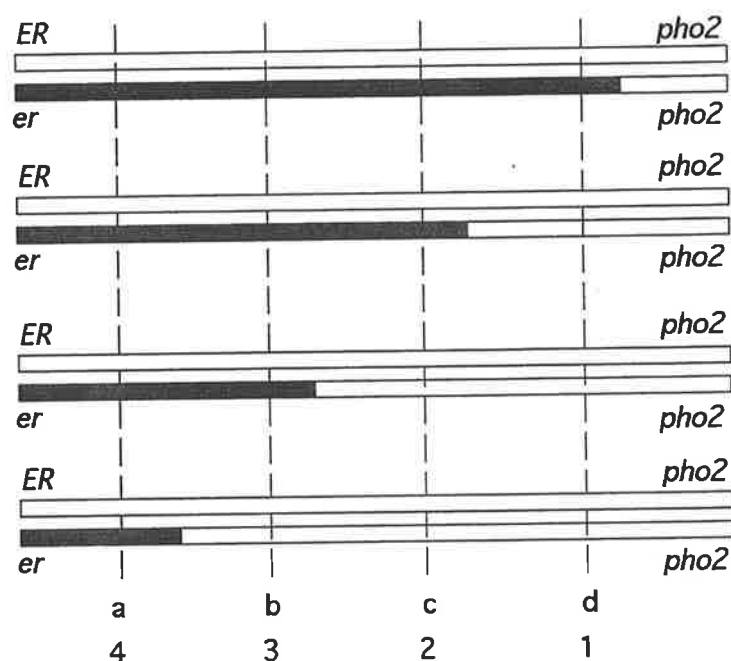
Fig. 5.9. Schematic diagram showing *ER/pho2* recombinants scored with different probes a, b, c and d. The numbers at the bottom indicate the number of heterozygotes scored by the corresponding probes. The probe closer to the *PHO2* locus, for example, probe "d" will score fewer heterozygotes than the probes closer to the *ER* locus, "a" and "b". The shaded bars represent the L.*er* background and the blank bars represent the Col background.

position 62.03 cM, Website: http://nase.nott.ac.uk/new_ri_map.html/) and eight showed a Col/L.*er* heterozygous pattern. The eight plants were further tested by the CAPS marker *cop1* (mapping position 62.56 cM, Website: http://nase.nott.ac.uk/new_ri_map.html/) and four were still Col/L.*er* heterozygous. The four recombinants were then tested by another CAPS marker *TEn5* (mapping position 69.7 cM, Website: http://weeds.mgh.harvard.edu/ goodman/c2_d.html) which is south of *cop1*, and in this case all plants were homozygous for the Col pattern. From these data, *PHO2* is expected to be located between *cop1* and marker T14G11 and is covered by the YAC clone CIC10F7 (Zachgo et al., 1996) which is 500 kb in length. The mapping data from both *AS1* and *ER* sides are combined and summarised in Fig. 5.10.
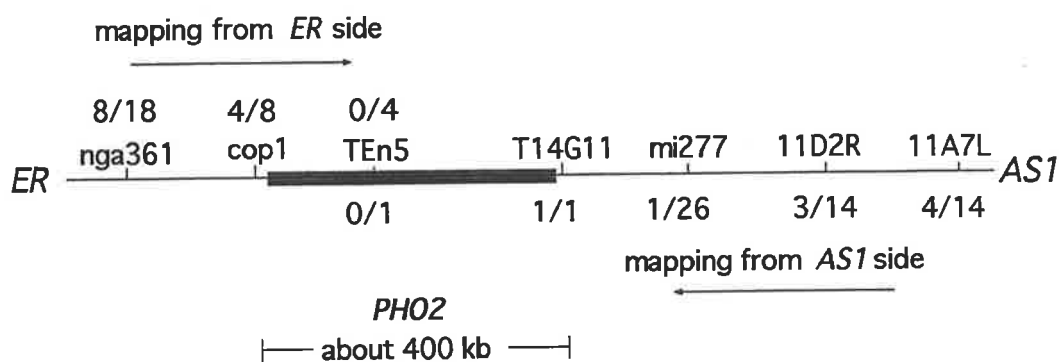
Fig. 5.10. Summary of the mapping data. The fractions indicate the number of recombinants with a homozygous Col pattern (from *AS1* side) or with a Col/L.er heterozygous pattern (from *ER* side) from the total number of the recombinants used for RFLP analysis. From *AS1* side, only the recombinant that showed a homozygous Col pattern at the marker *mi277* was tested further by markers T14G11 and *TEn5*. From the *ER* side, only the four plants showed Col/L.er heterozygous pattern at the marker *cop1* were tested further by the maker *TEn5*. The shaded area indicates the location of *PHO2*.

5.4. Discussion

Successfully cloning a gene by a map-based approach depends on accurate selection of recombinants, accuracy of the available genetic maps and good representation of DNA in

various libraries near the region of the gene of interest. One difficulty in mapping *PHO2* is

that the morphological phenotype of the *pho2* mutant is not always distinctive under all

growth conditions. To ensure the accurate screening of recombinants, all putative

recombinants were assayed at least once, but usually twice, for their Pi concentration and

those with a Pi concentration above 1.5 µg/mg fw were identified as *pho2*. This requirement

to assay plants for Pi has slowed the progress of mapping the gene.

PHO2 was mapped to a region between *cop1* and the marker T14G11 which corresponds to

a genetic distance of about 4 cM. Recent sequencing of chromosome 2 has defined a contig

of sequenced BAC clones which totally covers the region of *PHO2*. This contig spans *cop1*

and *mi277* (Website: http://genome-www3.stanford.edu/cgi-bin/AtDB/SeqRIMap?clone=

T14G11) and should therefore contain the *PHO2* gene. However, based on the protein

sequences encoded by the putative genes (about 70 genes) in the region and by comparing

these with proteins of known P-function from other organisms, there are no obvious

candidates for PHO2. It should be pointed out that a putative Pi transporter gene (identical to

PHT5, see Section 6.2), which is located on BAC T24L7 and BAC T21L14 on

chromosome 2, has been identified by genome sequencing. However, this gene is located to

the north of *cop1* and therefore, is clearly not the *PHO2* gene. More recombinants and other

molecular markers between *cop1* and the marker T14G11 can be used to find a smaller

region flanked by two markers such that a BAC clone containing *PHO2* can be identified.

This BAC clone could then be subcloned into a binary vector and used to transform the

pho2 mutant to assess whether a fragment is able to complement the *pho2* mutant. The

availability of plasmid vectors that can be used to transform plants with large pieces of DNA

such as BAC clones, makes this a feasible approach.

Once the *PHO2* gene is isolated, the spatial and temporal expression of the gene could be

determined by Northern blot analyses and by *in situ* hybridisations. Translation of the

coding sequence will reveal the amino acid sequence of PHO2 and comparison of this

sequence to proteins of known function may provide a lead for defining the function of

PHO2.

Chapter 6

General discussion

Understanding the molecular basis of Pi uptake and its regulation in plants will be facilitated by knowledge about the genes and their products responsible for the processes. Disrupting a single gene to generate a mutant with altered P nutrition and comparing the mutant to wild-type plants is one way to elucidate the function of genes. In this thesis, an EMS-mutated *Arabidopsis* mutant *pho2*, which accumulates Pi in leaves was used to study Pi uptake and transport by comparing it to wild-type seedlings. The study was aimed at defining the physiological lesions in *pho2* mutant and to obtain evidence regarding the function of the *PHO2* gene in P nutrition of higher plants.

6.1. *PHO2* is involved in regulation of P nutrition in shoot cells

Physiological work has shown that the uptake capacity of plant roots for many mineral nutrients is inversely related to their internal concentrations (Lee, 1982; Glass, 1983; Clarkson and Lüttge, 1991). Such a relationship between internal concentrations and influx has been demonstrated previously for P (Clarkson and Scattergood, 1982; Lefebvre and Glass, 1982; Katz et al., 1986). When the internal Pi concentration decreases, the uptake capacity increases several fold; as the internal concentration increases, the uptake rate declines. This so-called negative feedback regulation system may have evolved in plants to adjust their uptake capacity according to the plant's nutrient requirement to cope with a changing environment. The *pho2* mutation has somehow disturbed the negative-feedback regulation of Pi resulting in Pi accumulation in shoot cells. The mutation has also indirectly affected the Pi uptake capacity of roots which, in this study, has been demonstrated to be controlled by the shoot. Physiological characterisation of the *pho2* mutant suggests that the mutation could be in a gene encoding a Pi transporter, or a protein responsible for regulating Pi uptake. Mapping results showed *PHO2* is in a region of about 400 kb on chromosome 2 which has been totally sequenced. However, among the putative proteins in the region,

there is no Pi transporter. Therefore, *PHO2* is unlikely to encode a Pi transporter or, at least, does not encode a Pi transporter similar to the ones that have been characterised in plants to date. But it should be noted that all the currently cloned Pi transporter genes in *Arabidopsis* are primarily expressed in roots . It is possible that PHO2 is a Pi transporter that is related to Pi transport or translocation in shoot cells and has a different amino acid sequence to the root-expressed Pi transporters.

6.2. Phosphate transporter genes in *Arabidopsis* and other plant species

One of the prerequisites for understanding the molecular basis of P acquisition by plants is to clone the Pi transporter genes. Recently, five Pi transporter genes have been identified in the *Arabidopsis* genome based on sequence homology to the yeast Pi transporter gene *PHO84*. These are *AtPT1* (=APT2; PHT1), *AtPT2*, *APT1* (=PHT2), *AtPT4* (=PHT3) and *PHT5* (Muchhul et al., 1996; Smith et al., 1997; Lu et al., 1997; Mitsukawa et al., 1997a; Mitsukawa et al., 1997b). Figure 6.1 lines up the predicted amino acid sequences of the five Pi transporters from *Arabidopsis*. Unlike the relatively low homology of amino acid sequences with yeast (about 30-40% identity), these Pi transporters exhibit 75-99 % identity to one another. The predicted proteins consist of 12 membrane-spanning regions, which is a common feature of transporters responsible for various substrates such as ions, sugar, amino acids and antibiotics (Henderson, 1993; Marger and Saier, 1993). The predicted secondary structure of these proteins show similar conserved regions of three potential post-translation modification sites for protein kinase C, casein kinase II and N-glycosylation in the cytoplasmic side of the plasma membrane. The mapped Pi transporter genes in *Arabidopsis* are located on chromosome 5 or chromosome 2 (Mitsukawa et al. 1997a; Lu et al., 1997, Smith et al., 1997). Based on their chromosomal locations, none of these Pi transporter genes encode either *PHO1* or *PHO2* (Chapters 4 and 5)

Phosphate transporter genes isolated from other plant species include: *StPT1* and *StPT2* from potato (Leggewie et al., 1997); *PIT1* from *Catharanthus roseus* (Kai et al., 1997); and *LePT1* and *LePT2* from tomato (Liu et al., 1998). In mycorrhizal roots of *Medicago truncatula*, Pi transporter genes *MtPT1* and *MtPT2* (Liu et al., 1998) were also isolated. The

```
              10                  20                30
APT1   M A E Q Q L G V L K A L D V A K T Q L Y H F T A I V I A G M
APT2   M A E Q Q L G V L K A L D V A K T Q L Y H F T A I V I A G M
AtPT2  M A R E Q L Q V L N A L D V A K T Q W Y H F T A I I I A G M
AtPt4  M A D Q Q L G V L K A L D V A K T Q L Y H F T A I V I A G M
PHT5   M A K K G K E V L N A L D A A K T Q M Y H F T A I V I A G M

              40                  50                60
APT1   G F F T D A Y D L F C V S L V T K L L G R I Y Y F N P E S A
APT2   G F F T D A Y D L F C V S L V T K L L G R I Y Y F N P E S A
AtPT2  G F F T D A Y D L F C I S L V T K L L G R I Y Y H V E G A Q
AtPt4  G F F T D A Y D L F C V S L V T K L L G R L Y Y F N P T S A
PHT5   G F F T D A Y D L F S I S L V T K L L G R I Y Y H V D S S K

              70                  80                90
APT1   K P G S L P P H V A A A V N G V A L C G T L S G Q L F F G W
APT2   K P G S L P P H V A A A V N G V A L C G T L S G Q L F F G W
AtPT2  K P G T L P P N V A A A V N G V A F C G T L A G Q L F F G W
AtPt4  K P G S L P P H V A A A V N G V A L C G T L A G Q L F F G W
PHT5   K P G T L P P N V A A A V N G V A F C G T L A G Q L F F G W

              100                 110               120
APT1   L G D K L G R K K V Y G L T L I M M I L C S V A S G L S F G
APT2   L G D K L G R K K V Y G L T L V M M I L C S V A S G L S F G
AtPT2  L G D K L G R K K V Y G M T L M V M V L C S I A S G L S F G
AtPt4  L G D K L G R K K V Y G I T L I M M I L C S V A S G L S L G
PHT5   L G D K L G R K K V Y G I T L M L M V L C S L G S G L S F G

              130                 140               150
APT1   N E A K G V M T T L C F F R F W L G F G I G G D Y P L S A T
APT2   H E A K G V M T T L C F F R F W L G F G I G G D Y P L S A T
AtPT2  H E P K A V M A T L C F F R F W L G F G I G G D Y P L S A T
AtPt4  N S A K G V M T T L C F F R F W L G F G I G G D Y P L S A T
PHT5   H S A N G V M A T L C F F R F W L G F G I G G D Y P L S A T

              160                 170               180
APT1   I M S E Y A N K K T R G A F I A A V F A M Q G V G I L A G G
APT2   I M S E Y A N K K T R G A F I A A V F A M Q G V G I L A G G
AtPT2  I M S E Y A N K K T R G A F V S A V F A M Q G F G I M A G G
AtPt4  I M S E Y A N K K T R G A F I A A V F A M Q G V R I L A G G
PHT5   I M S E Y A N K K T R G A F I A A V F A M Q G F G I L A G G

              190                 200               210
APT1   F V A L A V S S I F D K K F P A P T Y A V N R A L S T P P Q
APT2   F V A L A V S S I F D K K F P A P T Y A V N R A L S T P P Q
AtPT2  I F A I I I S S A F E A K F P S P A Y A D D A L G S T I P Q
AtPt4  F V A L A V S S I F D K K F P S P T Y E Q D R F L S T P P Q
PHT5   I V S L I V S T F D H A F K A P T Y E V D P V G S T V P Q
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                      220                 230                   240
APT1   V D Y I W R I I V M F G A L P A A L T Y Y W R M K M P E T A
APT2   V D Y I W R I I V M F G A L P A A L T Y Y W R M K M P E T A
AtPT2  A D L V W R I I L M A G A I P A A M T Y Y S R S K M P E T A
AtPt4  A D Y I W R I I V M F G A L P A A L T Y Y W R M K M P E T A
PHT5   A D Y V W R I V L M F G A I P A L L T Y Y W R M K M P E T A

                      250                 260                   270
APT1   R Y T A L V A K N I K Q A T A D M S K V L Q T D I E L E E R
APT2   R Y T A L V A K N I K Q A T A D M S K V L Q T D I E L E E R
AtPT2  R Y T A L V A K D A K Q A A S D M S K V L Q V E I E P E Q Q
AtPt4  R Y T A L V A K N I K Q A T A D M S K V L Q T D L E L E E R
PHT5   R Y T A L V A R N T K Q A A S D M S K V L Q V D L I A E E E

                      280                 290                   300
APT1   V E D D V K D P R - - Q N Y G L F S K E F L R R H G L H L L
APT2   V E D D V K D P K - - Q N Y G L F S K E F L R R H G L H L L
AtPT2  K L E E I S K E K S - K A F G L F S K E F M S R H G L H L L
AtPt4  V E D D V K D P K - - K N Y G L F S K E F L R R H G L H L L
PHT5   A Q S N S N S S N P N F T F G L F T R E F A R R H G L H L L

                      310                 320                   330
APT1   G T T S T W F L L D I A F Y S Q N L F Q K D I F S A I G W I
APT2   G T T S T W F L L D I A F Y S Q N L F Q K D I F S A I G W I
AtPT2  G T T S T W F L L D I A F Y S Q N L F Q K D I F S A I G W I
AtPt4  G T T S T W F L L D I A F Y S Q N L F Q K D I F S A I G W I
PHT5   G T T T T W F L L D I A Y Y S S N L F Q K D I Y T A I G W I

                      340                 350                   360
APT1   P K A A T M N A T H E V F R I A R A Q T L I A L C S T V P G
APT2   P K A A T M N A T H E V F R I A R A Q T L I A L C S T V P G
AtPT2  P P A Q S M N A I Q E V F K I A R A Q T L I A L C S T V P G
AtPt4  P K A A T M N A I H E V F K I A R A Q T L I A L C S T V P G
PHT5   P A A E T M N A I H E V F T V S K A Q T L I A L C G T V P G

                      370                 380                   390
APT1   Y W F T V A F I D T I G R F K I Q L N G F F M M T V F M F A
APT2   Y W F T V A F I D T I G R F K I Q L N G F F M M T V F M F A
AtPT2  Y W F T V A F I D V I G R F A I Q M M G F F M T V F M F A
AtPt4  Y W F T V A F I D I I G R F A I Q L M G F F M M T V F M F A
PHT5   Y W F T V A F I D I L G R F F I Q L M G F I F M T I F M F A

                      400                 410                   420
APT1   I A F P Y N H W I K P E N R I G F V V M Y S L T F F F A N L
APT2   I A F P Y N H W I K P E N R I G F V V M Y S L T F F F A N F
AtPT2  L A I P Y N H W T H K E N R I G F V I M Y S L T F F F A N F
AtPt4  I A F P Y N H W I L P D N R I G F V V M Y S L T F F F A N F
PHT5   L A I P Y D H W R H R E N R I G F L I M Y S L T M F F A N F
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                  430            440            450
APT1    G P N A T T F I V P A E I F P A R L R S T C H G I S A A A G
APT2    G P N A T T F I V P A E I F P A R L R S T C H G I S A A A G
AtPT2   G P N A T T F V V P A E I F P A R F R S T C H G I S A A S G
AtPt4   G P N A T T F I V P A E I F P A R L R S T C H G I S A A T G
PHT5    G P N A T T F V V P A E I F P A R L R S T C H G I S A A S G

                  460            470            480
APT1    K A G A I I G A F G F L Y A A Q N Q D K A K V D A G Y P P G
APT2    K A G A I V G A F G F L Y A A Q S Q D K P K V D A G Y P P G
AtPT2   K L G A M V G A F G F L Y L A Q N P D K D K T D A G Y P P G
AtPt4   K A G A I V G A F G F L Y A A Q P Q D K T K T D A G Y P P G
PHT5    K A G A I V G A F G F L Y A A Q S S D S E K T D A G Y P P G

                  490            500            510
APT1    I G V K N S L I V L G V L N F I G M L F T F L V P E P K G K
APT2    I G V K N S L I M L G V L N F I G M L F T F L V P E P K G K
AtPT2   I G V R N S L I V L G V V N F L G I L F T F L V P E S K G K
AtPt4   I G V K N S L I M L G V I N F V G M L F T F L V P E P K G K
PHT5    I G V R N S L L M L A C V N F L G I V F T L L V P E S K G K

                  520            530            540
APT1    S L E E L S G E A E V S H D E - - - - - - - - - K
APT2    S L E E L S G E A E V S H D E - - - - - - - - - K
AtPT2   S L E E M S G E N E D N E N S - - - - - - - - N N D S R T V
AtPt4   S L E E L S G E A E V D K
PHT5    S L E E I S R E D E Q S G G D T V V E M T V A N S G R K V

                  550            560            570
APT1
APT2
AtPT2   P I V
AtPt4
PHT5    P V
```

Fig. 6.1. Deduced amino acid sequences of the five Pi transporter genes from *Arabidopsis*. The shading indicates identical amino acids while the boxed amino acids indicate similar ones. The accession number of each gene is: *APT1*(=*PHT2*)--Y07681; *APT2* (=*AtPT1*, *PHT1*)--Y07628; *AtPT2*--62331; *AtPT4* (=*PHT3*)--U97546; *PHT5*--AB000093.

functional characteristics and expression pattern of these genes are consistent with a role in the acquisition of Pi from the environment rather than Pi transport at the symbiotic interface in mycorrhizal roots. Generally, these Pi transporter genes are expressed most strongly in roots, except for *StPT1* which is also expressed in other tissues.

The existence of multiple genes that encode Pi transporters in roots is consistent with physiological studies that Pi taken up from environment into the plant involves different types of cells. These cells, including epidermal cells, cortical cells and stellar cells may have specific Pi transporters which differ between the cell types. Further *in situ* hybridisation work with these genes and their products may provide the location and functions of these Pi transporters. The existence of multiple Pi transporters is also consistent with kinetic studies that showed more than one uptake system involved in Pi acquisition by roots (Epstein, 1976; Dunlop et al., 1997). Similarly, in leaf cells, the presence of multiple Pi transporters could be expected although to date there are no reports of shoot-specific forms. These transporters could be responsible for the transport of Pi between different compartments, for example, from the apoplast into the cytosol and from the cytosol into vacuoles or vice versa. In addition, the expression of these genes could be constitutive or some of them may be induced by Pi stress.

6.3. Pi regulation in higher plants

Compared with microorganisms where the Pi-scavenger system consisting of a range of genes has been well-characterised, Pi regulation in higher plants is far from being understood. In addition to the progress of molecular studies on Pi uptake studies which constitutes only a part of P nutrition, other genes related to P nutrition have also been isolated. In *Arabidopsis*, ribonuclease genes *RNS1* and *RNS2*, whose transcripts were greatly and specifically induced by P starvation, were demonstrated to be regulated by P nutrition (Taylor et al., 1993; Bariola et al., 1994). The RNase enzymes presumably degrade macromolecules of RNA in senescing cells, freeing the Pi for remobilisation to reproductive structures (Kelly and Davies, 1988). It is possible that they also participate in remobilisation of Pi in *Arabidopsis* when Pi is limiting. Additional cDNA clones with

expression levels increased by P starvation in different plant species were also reported. In tobacco cultured cells, these genes either showed homology to the *partB* gene in tobacco whose product has glutathione S-transferase (GST, EC 2.5.1.18) activity or did not show homology to previously cloned genes (Ezaki et al., 1995). In *Brassica nigra* cell cultures, a gene encoding a product homologous to β-glucosidase (EC 3.2.1.21) was also reported (Malboobi and Lefebvre, 1995). Plant β-glucosidase are implicated in several growth-related functions such as abscisic acid metabolism (Matsuzaki and Koiwai, 1986), cell wall metabolism (Taiz, 1984) and catabolism of β-glucosides of various flavones (Hosel and Conn, 1982). The role of β-glucosidase in P-starved plants cells remains to be determined. It could be involved in the deglycosylation and hence, regulation of phosphatases and other enzymes during P-stress (Gellatly et al., 1994). From tomato, Liu and Raghothama (1995) isolated a cDNA (*TPSI1*) indued by Pi-starvation using differential display. They found that the *TPSI1* transcripts were rapidly induced in both roots and leaves during Pi starvation and that the transcripts decreased when P-starved plants were resupplied with Pi (Liu et al., 1997). Southern blot analysis showed that there is only a single copy of *TPSI1* in the tomato genome. Similar genes are also present in other plant species including potato and tobacco suggesting a general role in plants. The promoter region of *TPSI1* contains several conserved sequences found in Pi-starvation induced genes of yeast (Liu et al., 1997). These results suggest that *TPSI1* may be involved in the early response to Pi starvation in plants.

Evaluation of these data and by analogy to the Pi-regulated system in yeast, a P-starvation-induced system may also exist in plant. According to this system, Pi as a repressor, may interact with sensor proteins that in turn control a number of genes. This could result in the down-regulation of the expression of genes encoding phosphatases, Pi transporters, ribonucleases and other yet-to-be identified proteins (Fig. 6.2). Changes in root growth and architecture which is possibly related to the alteration of plant hormones (Masucci and Schiefelbein, 1994) are also attributed to adaptation of plants to Pi stress (Caradus et al., 1993; Lynch 1995) and probably controlled by the system. In this hypothetical system, *PHO2* of *Arabidopsis* is unlikely to be analogous to *PHO4* in yeast (see Section 1.6.1) which activates a range of Pi-repressible genes encoding acid phosphatase and high-affinity
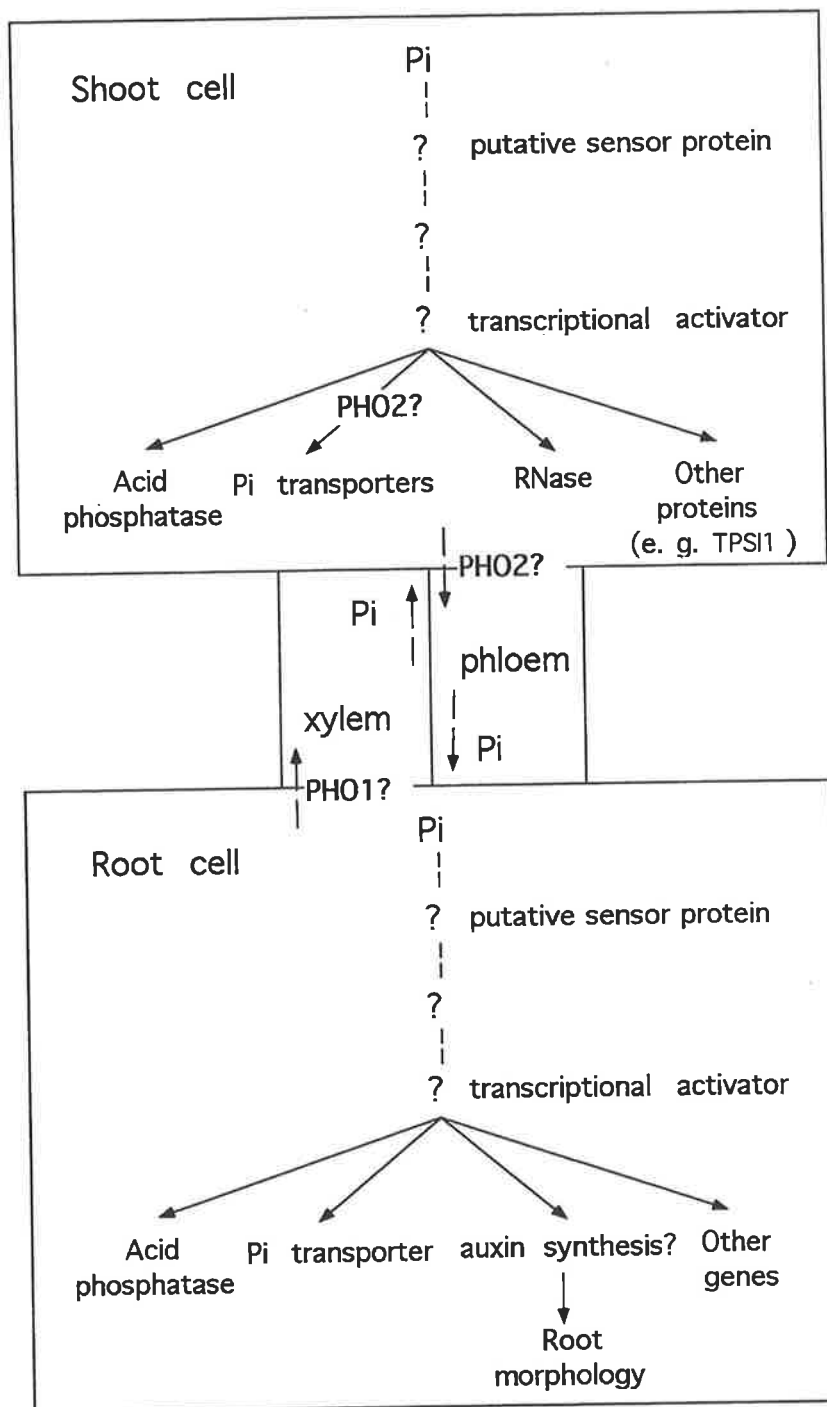
Fig. 6.2. Hypothetical scheme of the main proteins and processes regulated by P nutrition in higher plants. The question marks and the dashed lines represent unknown genes and their regulation (activation or inhibition) in a Pi signal transduction pathway. The arrows denote the proteins or processes being repressed by Pi. The dashed arrows indicate the Pi flow between shoot and root, which may regulate P nutrition in the whole plant. Possible positions of PHO1 and PHO2 in these processes are shown.

Pi transporter under P-starvation because the *pho2* mutant shows normal activity of acid-phosphatase and therefore, is not a "de-repressed mutant" (Section 2.3.3). *PHO2* appears to specifically affect either Pi uptake in shoot cells or Pi export from shoots to roots. Similarly, *PHO1* may only affect loading of Pi in the xylem because the uptake capacities of roots and leaves are similar to that of wild-type plants (Poirier et. al., 1993).

As a multi-cellular organism, regulation of P homeostasis in plants can be expected to be more complicated than in microbes due to the greater structural complexity. Phosphate transport between different cells and changes in Pi concentration in particular tissues could affect other organs. In the *pho1* mutant, for example, the defect in loading of Pi into the xylem in the root results in Pi deficiency in leaves, and therefore results in increased activity of acid phosphatase and probably Pi uptake by leaf cells. In the *pho2* mutant, deregulation of Pi in leaf cells has also affected the capacity of Pi uptake by roots.

6.4. Further work on the *pho2* mutant

Further physiological work on the *pho2* mutant could focus on determining the location of excessive Pi accumulation in shoot cells by comparison to the wild-type plants. ^{31}P-NMR is an ideal approach as it discriminates the Pi concentration in vacuoles from cytoplasmic Pi directly based on the difference in pH of these compartments. Measuring the Pi concentration in the isolated vacuoles from leaf cells may also be successful by improvement of the method of isolation. In addition, ^{32}Pi uptake by leaf protoplasts could be conducted to determine whether the leaf cells of *pho2* mutants have greater uptake capacity which could account for the excessive accumulation of Pi. These studies may help to provide information on whether Pi transport across the plasma membrane or tonoplast is affected in the *pho2* mutant. At the whole plant level, analysis of phloem sap to determine the species of P and their concentrations under different P-nutritions is another line of study. This may provide evidence regarding the role of mobile P forms in controlling Pi uptake by roots.

Cloning the *PHO2* gene and investigating its expression will offer further insight into its functions. In the present study, *PHO2* has been mapped to a completely sequenced region

that is covered by several BAC clones. To date, the translation products of genes identified on BACs in the region of *PHO2* indicate no obvious candidate. There are a number of genetic markers available in this region to allow further fine mapping of *PHO2*. These data, along with information from the *Arabidopsis* genome sequencing, will form the basis for cloning the *PHO2* gene in the future.

Characterising other *Arabidopsis* P-nutrition mutants will help to elucidate the relationships among these genes and provide a better understanding of the Pi uptake process and its regulation in higher plants. Recently, an *Arabidopsis* mutant missing one acid phosphatase has been identified (Trull and Deikman, 1998). The mutant, designed *pup1,* is able to respond to P-deficient conditions by an increase in overall level of acid phosphatase activity. The mutant will be useful for determing the role of the missing acid phosphatase isoform in plants under P deficiency. As Pi uptake and regulation is expected to be complicated with many genes involved as already described, it is likely that additional mutants in P nutrition will be found in *Arabidopsis* in addition to the *pho1*, *pho2* and *pup1* mutants. Identifying mutants influencing Pi acquisition from the external medium or Pi allocation between cytosol and vacuoles will be helpful in understanding the underlying molecular mechanisms of Pi uptake. The approach of identifying "tagged" mutants by screening transposon or T-DNA insertion lines will also facilitate the cloning of genes involved in P nutrition. With this knowledge, in the longer term, molecular manipulation of economic crops to improve P nutrition would be possible.

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