Phosphate nutrition in the *Ricinus communis* L. seedling: Role of the phosphate transporter and acid phosphatase

Dissertation zur Erlangung des Doktorgrades

an der Fakultät für Biologie, Chemie und Geowissenschaften

der Universität Bayreuth

vorgelegt von

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aus Viet nam

Bayreuth, 2006

Vollständiger Abdruck der von der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigten Dissertation.

Tag der Einreichung: 15. Nov 2006

Tag des wissenschaftlichen Kolloquiums: 23. Februar 2007

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For my Family

Contents

List of Abbreviations

Amp	ampicillin
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
ß-ME	ß-mercaptoethanol
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphatase
DTT	dithiothreitol
DIG	digoxygenin
dd$H_2$0	deionized water
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EM	electron microscope
et al.	*et alii*
Fig	figure
h	hour
g	“gram”
His	histidin
Ig	immunoglobulin
IPTG	isopropyl-ß-D-thiogalactopyranoside
kD	kilo Dalton
L	litre
M	molarity
m	milli
min	minute(s)
mM	millimolar
mRNA	messenger ribonucleic acid

MS	Murashige Skoog medium
NBT	nitroblue tetrazolium
OD	optical density
ORF	open reading frame
ori	origin of replication
Pi	inorganic phosphate
PBS	phosphate- buffer saline
PCR	polymerase chain reaction
PFA	paraformaldehyd
PMSF	phenylmethansulfonylfluorid
pNPP	p-nitrophenylphosphate
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	sodium citrate (buffer)
sec.	seconds
TBE	Tris/borate/EDTA (buffer)
TE	Tris/ EDTA (buffer)
u	unit
UV	ultraviolet
Vol.	volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

1 Introduction

Phosphate (Pi) is one of essential macronutrients required for plant growth and development. It severs as an essential component of macromolecules such as nucleic acids and phospholipids. Furthermore, Pi plays an important role in various metabolic processes, such as photosynthesis, respiration, energy conservation, membrane synthesis, carbohydrate metabolism and signal transduction. In the natural ecosystem, the growth of plants is controlled by the availability of Pi. Although Pi occurs in high concentrations in soil it presents on insoluble mineral forms which is not available for plant uptake and utilization. In most agricultural systems, every year many million metric tons of Pi fertilizers have been applied to the soil to promote plant growth but more than 80% of the Pi in the soil are converted into an immobile and unavailable form due to adsorption, precipitation or formation of the organic form. Twenty to eighty percent of Pi in the soil is found in organic form which needs to be mineralized to the inorganic form before it becomes available for plants (Richardson, 1994; Holford, 1997). In fact, the available Pi concentrations in soil solutions are often less than 10 µM (Bielski, 1973). Pi moves through the soil mainly by diffusion, but the rate of diffusion is slow (10^{-12} to 10^{-15} m^2 s^{-1}) therefore a depletion zone of Pi around plant roots is caused. Due to the inefficient uptake of Pi fertilizers by plants, excessive Pi may run off into surface water thereby polluting aquatic ecosystems and contributing to the process of eutrophication. Moreover, the natural source of inorganic Pi fertilizers, such as phosphate rocks, are expected to be depleted over next 60 to 90 years and Pi availability to plants will be a great matter in the future (Hammond *et al*., 2004). Thus, genetically modified plants efficient in Pi uptake may be an alternative to resolve the phosphate limitation problem. Elucidation of the complex mechanisms of plants acclimation to Pi starvation is the basis for efficient crop breeding.

1.1 Morphological plant responses to Pi starvation

Pi limitation results in a decrease of the photosynthesis rate and of stomata conductance (Clarkson *et al*., 1982). To avoid or ameliorate this basically growth-retarding effect, plants grown under Pi starvation conditions develop numerous responses, such as morphological, physiological, biochemical and molecular reactions. Modification of root growth results in change of root geometry and morphology, which is the first major

response to Pi starvation (Lynch, 1995). The root mass increases, while root diameter decreases. Increased root growth has been reported in several plants, such as spinach, onion, rape, tomato and bean (Fohse, 1988). Furthermore, Bates and Lynch (1996) showed that Pi starvation also elicits root hair growth in *Arabidopsis* plants by nearly 3-fold. Another result from rye grown under Pi starvation showed that root hairs contributed for up to 63% of total Pi uptake (Gahoonia *et al*., 1998). In addition there is a correlation between mycorrhiza formation and Pi status in soil. Fungi colonize the root cortex and obtain carbon from the root, concurrently enhancing the Pi acquisition in plants (Harrison, 1999). In non-mycorrhizal plants, such as white lupin, proteoid roots are formed in response to Pi starvation. Proteoid roots are branched in bottle-brush like clusters of rootlets which are covered with abundant root hairs. These roots function in synthesis and secretion of organic acids thus liberating phosphates to the rhizophere. Concurrently, proteoid roots also absorb Pi quicker than non-proteoid roots (Gardner *et al*., 1982; Johnson *et al*., 1996; Keerthisinghe *et al*., 1998). The studies *Arabidopsis* plants under Pi starvation showed plants exhibiting Pi starvation symptoms such as accumulation of anthocyains, stunted shoots with small dark green leaves and increased production of Pi acquisition enzyme (Green, 1994; Bates and Lynch, 1996; Trull *et al*., 1997).

1.2 Biochemical and genetic responses to Pi starvation

1.2.1 Pi transport mechanism in plants

Plants take up phosphorus in the orthophosphate (Pi) forms $H_2PO_4^-$ and HPO_4^- , which are present depending on pH. The dissociation of H_3PO_4 into $H_2PO_4^-$ has a pK of 2.1 the while the dissociation of H_3PO_4 into HPO_4^- occurs with a pK of 7.2. Various reports suggested that Pi uptake rates in plants are highest among pH 5.0 and 6.0 (Ullrich-Eberius *et al*., 1984; Furihata *et al*., 1992) and monovalent $H_2PO_4^-$ is mostly transported into plant cells through the plasma membrane. Adding Pi to starved roots results in depolarization of plasma membrane and acidification of the cytoplasm (Ullrich and Novacky, 1990). This suggests that Pi is co-transported with at least two positively charged ions such as proton and two to four protons are supposed to be taken up with each monovalent $H_2PO_4^-$ (Ullrich-Eberius *et al*., 1981; Sakano *et al*., 1990). Phosphorus

is taken up by plants via the root to the xylem; Pi is moved from the xylem to cytoplasm and subsequently moved to vacuole. Because of the difference between external (micromolar range) and internal Pi concentration (millimolar range) and the net negative charge on the inside of the plasma membrane, this movement of Pi has been proposed against the steep electrochemical potential gradient that requires energized transport (Ullrich and Novacky, 1990). Although a H^+-coupled Pi transport system was recognized in plants, recently, Reid and colleagues (2000) have reported a Na^+-coupled Pi transport system that functions in the green alga *Chara coralline* under Pi starvation.

Numerous reports on the analysis of Pi uptake kinetics in plants under different Pi concentrations have mentioned the existence of two Pi transport systems, one is a high affinity system active at low external concentrations with K_m values of 3 to 10 μM and another is a low affinity system active at high external Pi concentrations with K_m values of 50 to 330 μM (Ullrich-Eberius *et al*., 1981; Furihata *et al*., 1992; Schmidt *et al*., 1992; Mimura *et al*., 1998). Due to the often low level of Pi in soil solution (less than 10 μM), it is proposed that mostly the high-affinity system is responsible for Pi uptake in plants. While the high-affinity system is regulated by the availability of Pi, the low-affinity system is expressed constitutively in plants. However, the high- and low- affinity Pi transport systems have been also found in bacteria (Harris *et al*., 2001), yeast (Persson *et al*., 1998) and mammals (Olah *et al*.,1994).

Several reports have shown that the movement of Pi along plants after uptake into root symplasm results in distribution of Pi in whole plants (Mimura *et al*., 1996; Jeschke *et al*., 1997). In Pi sufficient plants, most of the Pi absorbed by the root is transported in the xylem to the young leaves, while re-translocation of Pi from older leaves to the roots through shoots occurs during Pi mobilization. In Pi deficient plants, the limited supply Pi is supplemented by mobilizing stored Pi in older leaves and re-translocation to both the younger leaves and growing root. This process involves both the depletion of Pi stores and the breakdown of organic Pi (Jeschke *et al*., 1997).

1.2.2 Pi regulated gene expression

During last decade, many Pi starvation inducible genes have been identified from plants such as high-affinity phosphate transporters, acid phosphatases and ribonucleases genes. This suggests that the genes are involved in response to Pi starvation probably

share a common regulatory system and have a co-ordination of gene expressions. The regulatory network, a phosphate starvation regulon (PHO), that controls the adaptation of plants to Pi starvation, is supposed to be similar to that which has been well studied in bacteria and yeast (Goldstein, 1992; Raghothama, 1999). Oshima and colleagues (1996) proposed that the PHO-regulon in yeast represents a complex multigene system and responses to Pi starvation by inducing the production of three acid phosphatases (Pho5, Pho10, Pho11) and a high-affinity phosphate transporter (Pho84). The members of PHO-regulon, including positive regulators (Pho2, Pho4, Pho81) and negative regulators (Pho80, Pho85) play as the key factors in controlling expression of Pi transporter and acid phosphatase genes.

The Pho80/Pho85 cyclin-cyclin dependent kinase (CDK) complex controls the phosphorylation of Pho4, but itself is controlled by the Pho81. Under Pi starvation, Pho81 protein inhibits Pho80-Pho85 cyclin-cyclin dependent protein kinase (CDK) complex, thus repressing phosphorylation of Pho4. Subsequently, the Pho4 is imported into nucleus and interacts with a second transcription factors (Pho2) and binds to the promoter of *pho* genes thereby activating transcription of the respective genes (Lenburg and O´Shea, 2001). By contrast, under Pi availability, the Pho80/Pho85 complex phosphorylates Pho4 at five serine residues, causing Pho4 inactivation thereby preventing its nuclear localization, thus terminating expression of Pi starvation inducible genes (O´Neill *et al*., 1996).

Recently, a Pi starvation mutant of the unicellular photosynthetic alga *Chlamydomonas reihardtii* defective in a number of specific Pi starvation responses, *psr1* line (phosphorus starvation response), was identified; that showed inability to activate expression of high-affinity Pi transporters and acid phosphates genes under Pi starvation. Rubio and colleagues (2001) have found *PHR1*, a similar gene to *PSR1,* in *Arabidopsis thaliana*. This suggests the existence of similar regulatory systems in the response to Pi starvation in algae and higher plants. In addition, several *Arabidopsis thaliana* mutants defective in a number of specific Pi starvation responses were found. For example, the lack of ability to load Pi into the xylem has been found in *pho1* mutants of *Arabidopsis*, suggesting the existence of Pi transporters involved in xylem loading within roots (Poirier *et al*., 1991). In addition, *pho3* mutants of *Arabidopsis* exhibit a number of defective responses to Pi starvation, such as no increasing APase activity, reduction of the Pi

accumulation in roots and shoots in Pi sufficient condition, reduced growth and accumulation of starch. The results provide the evidence for a lack of regulatory components of the PHO-regulon in the *pho3* mutant (Zakhleniuk *et al*., 2001). Despite numerous Pi starvation inducible genes have been identified in plants, the information for a hypothetical plant PHO-regulon is still fragmentary.

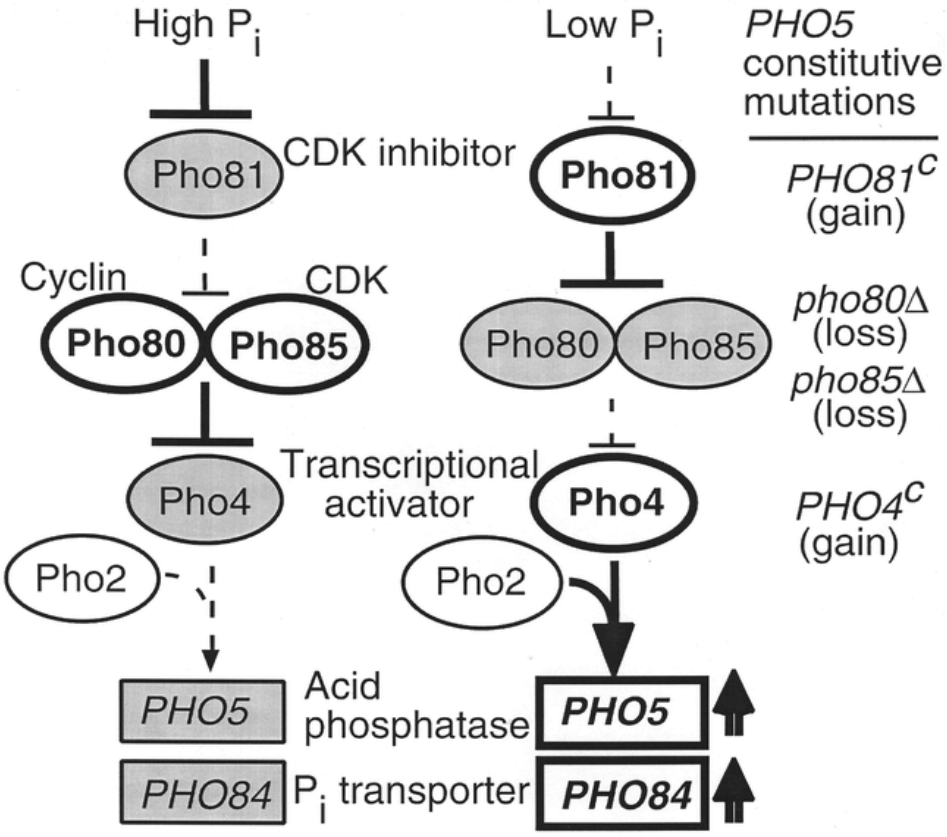


Figure 1.1: Regulation of gene expression in the PHO-regulon of *S. cerevisiae*. Regulator proteins and genes are presented as ovals and boxes, respectively. Thick lines mean that the signals are transduced to the downstream component, while dotted lines indicate the absence of an interaction with downstream components. Open ovals and boxes indicate activity states, gray ovals and boxes indicate an inactive state (taken from Ogawa *et al*., 2000).

1.2.3 Regulation of plant phosphate Transporters

Since the first high-affinity phosphate transporter, *PHO84*, was isolated from yeast (Bunya *et al*.,1991), many phosphate transporter genes have been isolated from plants, such as potato (*StPT1* and *StPT2*) (Leggewie *et al*., 1997), *Arabidopsis* (*AtPT1* and *AtPT2*) (Muchhal *et* al.,1996; Smith *et al*.,1997), *Medicago truncatula* (*MtPT1* and *MtPT2*) (Liu *et al*., 1998), tomato (*LePT1* and *LePT2*) (Daram *et al*., 1998; Liu *et al*.,1998a), *Nicotiana tabacum* (*NtPT1*) (Baek *et al*., 2001) and *Oryza sativa* (*OsPT11*) (Paszkowski *et al*., 2002). The gene sequences share high similarity with the high-affinity phosphate transporter sequence from yeast. All the cloned phosphate transporters are integral membrane proteins that contain 12 membrane spanning regions, which are separated into two groups by a large hydrophobic region. This feature has also been seen in other proteins involved in transport of sugars, amino acids and ions. They all belong to the "Major Facilitator Super family" (MFS) of transporters (Pao *et al*., 1998).

The enzymatic activities of expressed phosphate transporter genes were analyzed by functional yeast complementation of the yeast mutant *pho84*, which is deficient in high-affinity Pi uptake (Muchhal *et al*., 1996; Leggewie *et al*., 1997; Daram *et al*., 1998) and by genes expressed in cultured tobacco cells in Pi deficient media (Mitsukawa *et al*., 1997). Plant phosphate transporters have a pH optimum of 4.5 to 5.0 in the yeast expression system suggesting preferential uptake of $H_2PO_4^-$ by Pi transporters.

Almost all phosphate transporter genes are expressed when plants grow under Pi starvation, but their spatial expression patterns are different. Some Pi transporters are expressed in distinct tissues; *in situ* hybridization revealed *LePT1* is not only expressed in the roots but also expressed palisade parenchyma and phloem cells of leaves (Liu *et al*., 1998a). *StPT1* is expressed in various tissues, including root, flowers and tubes (Leggewie *et al*., 1997). *MtPT1* is expressed in the plasma membrane of root hairs and epidermal cells, indicating that these genes are responsible for Pi uptake by the root (Chiou *et al*., 2001). Furthermore, the transcription of *LePT1* and *LePT2* were decreased when resupplying Pi into Pi starved plants; probably, there is coordination between gene expressions, which is depended on the Pi status. This result provided more evidence of the role of Pi transporters in the uptake of Pi under Pi starvation conditions (Daram *et al*., 1998).

The phosphate transporters in plants have been classified into two families named *Pht1* and *Pht2*. Member of the *Pht1* transporter family, including most of the published sequences, differ from those of the *Pht2* transporter family in primary structure, affinity for Pi and in the presumed function. The members of the *Pht1* family of plant phosphate transporters are particularly expressed in roots, while *Pht2* transporters are expressed in both roots and shoots (Bucher *et al*., 2001). The first member of *Pht2* family, *ARAth;Pht2;1* was isolated from *Arabidopsis* cDNA library. Pht2;1 transporters has twelve membrane spanning domain as Pht1 family, but Pht2;1 transporters have a large hydrophilic loop between membrane spanning domain number eight and nine, while Pht1 transporters have that in membrane spanning domain number six and seven. Although, Pht2;1 protein sequence is highly similar to that of eukaryotic sodium dependent Pi transporters, functional analysis of the Pht2;1 protein in mutant yeast showed that it is a H+/Pi symporter; moreover its fairly high apparent K_m for Pi (0.4 mM) (Daram et al., 1999).

1.2.4 Acid phosphatases

Phosphatases have been classified as alkaline phosphatases or acid phosphatases (APase) based on their optimal pH for catalysis. Alkaline phosphatases hydrolyze phosphoesters at pH optima above 7.0 and typically display strong substrate specificity. Acid phosphatases hydrolyze orthophosphate esters and have pH optima below 6. These enzymes are ubiquitous in plants and are supposed to be involved in the release and recycling of Pi. In plants, APases can be detected through all developmental stages, in different tissues and in cell wall-associated forms. Moreover, APases have been found to function in response to Pi starvation, salt stress and water deficit (Duff *et al*., 1994; Granjeiro *et al*., 1999). According to their relative substrate specificities, plant APases can be divided into two types; one very specific for its substrate, the second type non-specific. Two large groups of specific APase, the phytases and the protein phosphatases, have been studied intensively. Phytases hydrolyze phytic acid to Pi, for example during seed germination. Protein phosphatases catalyze the removal of phosphates from tyrosine, serine and threonine residues of proteins.

Non-specific APases are widely distributed in a variety of plants and in many tissues. They exist in the form of monomeric or dimeric glycoproteins with subunit molecular weights of 30-65 kD. Both intra-cellular and extra-cellular APases are involved in many

physiological processes. Intra-cellular APases have been found in the vacuole and cytoplasm, while extra-cellular APases occur in the root apoplast and the medium of plant cell suspension cultures. Although the function of extra-cellular APases needs to be further characterized, several reports have confirmed that Pi starvation induces the secretion of these enzymes to the rhizosphere in several plant species, such as rice, tomato, wheat, soybean, lupin and maize. However, the secretion is different between species. The localization of extra-cellular APases in specific plant tissues was supposed to function in the liberation of Pi from organic sources in soil in order to maintain the Pi status for plant growth (Goldstein *et al.*, 1988a; Tadano *et al.*, 1991; Gilbert *et al*., 1999; Yun *et al*., 2001; Game *et al.*, 2001). Lee (1988) showed that extra-cellular APases are mainly localized in apical meristems and the outer surface of root epidermal cells. Concurrently, extra-cellular types from *Brassica nigra* suspension cells are localized in the cell wall (Lefebvre *et al*., 1990).

Intra-cellular APases are supposed to be involved in Pi remobilization from vacuolar or cytoplasmic phosphate stores. Schachtman and colleagues (1998) reported that in order to maintain the Pi status during Pi starvation, the plants remobilize Pi from metabolically less active sites in the cell, such as vacuoles, to metabolically more active sites, such as cytoplasm. Moreover, vacuolar APases also have been assumed to be involved in Pi remobilization from senescent plant tissues to feed young tissues; parallel to the fact that Pi starvation often enhances senescence in leaves (Snapp *et al*., 1996). Intra-cellular APases have been found in various plant organs and tissues, including leaves, shoots, flowers, fruits, germinating seeds, and cultured cells (Barker *et al*., 1974; Haraguchi *et al*., 1990; Duff *et al*., 1991a, 1991b; Ibrahim *et al*., 2002). There are many effectors which can be activators or inhibitors of plant APase expression. Several reports showed a relation of Pi status to synthesis and secretion of APases in plants (Lee, 1998; Duff *et al*., 1989b, 1991b; Lefebvre *et al*., 1990). Pi can be an inhibitor of APase by feedback action on APase activity. In fact, based on structural similarity with Pi, several effectors, including tartrate, vanadate and molybdate are considered as common inhibitors of APases. The effect of metallic cations on APases activities varied between plant species, developmental stage and APase type (Duff *et al*., 1989a; Granjero *et al*., 1999). The purified enzymes from leaves and nodules of *Phaseolus vulgaris* are greatly inhibited by Zn^{2+}, Hg^{2+}, Cu^{2+}, Pb^{2+}, Al^{3+} and molybdate and partially inhibited by Li^{+}, Mn^{2+}

and Co^{2+}. Turner and Plaxton (2001) found that banana APase activity was activated by Mn^{2+} and Mg^{2+}.

Although there are many reports on the involvement of APases in plant metabolism and APases responses to Pi starvation in plant roots, only few APase genes and peptide sequences from plants have been identified. Del Pozo and colleagues (1999) reported a type 5 acid phosphatase gene, *AtACP5*, isolated and characterized from *Arabidopsis. AtACP5* is 1014 bp long and contains an open reading frame (ORF) encoding a 338 amino acid polypeptide, including 31 amino acid N-terminal extension with characteristics of a signal peptide to cell membranes. The high expression of *AtACP5 in Arabidopsis* grown under Pi starvation was detected in roots, shoots and senescent leaves. The Pi supplement to Pi starved plants can reverse the induction of this gene. Moreover, the induction of *AtACP5* was also found in response to salt stress, oxidative stress and abscisic acid, whereas other phytohormones and other nutrient starvation, such as potassium or nitrogen did not induce expression of *AtACP5*. In addition, transgenic *Arabidopsis* plants containing an *AtACP5*: β-glucuronidase (GUS) fusion also showed high activity in response to the above-mentioned stresses. These results suggest that *AtACP5* plays a role in internal remobilization of Pi in plants.

Another APase gene isolated from tomato (*Lycopersicon esculentum*), *LePS2*; is 942 bp long and contains an open reading frame (ORF) encoding a 269 amino acid polypeptide, that shares high similarity to two distinct peptides, HAD and DDDD, from other members of the super families of phosphohydrolases. Under Pi starvation, expression of *LePS2* is rapidly induced in all tissues of tomato plant. Similarly, induction of *LePS2* transcription appears to be strong in tomato cell cultures after 3 hours of growing in Pi deficient conditions (Baldwin *et al*., 2001). Although, as *AtACP5*, expression of *LePS2* is also repressed when Pi is added to Pi starved plant. The expression of *LePS2* showed no expression in other nutrient stresses. The expression of *LePS2* genes was also identified in tomato plants during flowering under normal (Pi sufficient) growth condition and after pathogen infection.

In *Arabidopsis*, a gene for secreted APase, *AtsAPase*, was isolated (Haran *et al*., 2000). This gene of 1380 bp encodes a peptide of approximately 450 amino acids and 46 kD. This gene was induced in roots under Pi starvation. *AtsAPase* promoter was fused to

green fluorescent protein (GFP) as a reporter and the signal GFP was observed in root exudates of Pi starved plants.

Recently, three members of the *LePS2* gene family of acid phosphatases were isolated from a cDNA library of tomato (*Lycopersicon esculentum*) cell cultures growing in media lacking Pi. Stenzel and colleagues (2003) reported the nucleotide sequence of these three genes, *LePS2A*, *LePS2B*, *LePS2C*, having high similarity except for their C-terminal sequences and their 3´ untranslated regions. The expression of these three genes in Pi starved cell cultures and in Pi starved plants is similar to that of the *LePS2* gene. However, during the first few days of germination of seedlings, the authors have found that these three genes are expressed in the same level in both conditions, with Pi or deficient in Pi.

1.3 Phosphates in germinating seeds.

Phosphates play a vital role in a variety of reactions in seeds, such as formation of various phosphate sugars and nucleotides for metabolism and energy producing processes and synthesis of nucleic acids. Phosphorus is stored in seeds to a small part as inorganic orthophosphate and mostly in the organic form as phospholipids, phosphate ester of sugars, nucleic acids and nucleotides and especially phytin, a mixed cation salt of *myo*-inositol hexaphosphoric acid (phytic acid) which constitutes 60 to 80% of the total phosphate content of the seed (Lott *et al*., 1995). Phytin is concentrated in inclusion bodies (globoids) inside of the protein bodies of the storage tissue cells. While potassium and magnesium are typical counter ions in these salts, other mineral cations found in these globoids are calcium, manganese, zinc, barium and iron (Lott *et al*., 1982; Greenwood and Bewley, 1984). In rice (*Oryza sativa*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), most of phytic acid is found in the endosperm and in the aleurone layer, whereas in the yellow lupine (*Lupinus luteus*) and cotton seed phytin is in a sub-epidermal layer of the cotyledon. In castor bean (*Ricinus communis*) phytin deposits are mainly in the endosperm (Organ *et al*., 1998). Phytin is degraded during germination by phytase, a specific enzyme, which belongs to the family of histidine acid phosphatases. This enzyme hydrolyzes phytic acid to inositol and free orthophosphate and liberates mineral cations, which are essential for the germination and growth of

seedlings. Inorganic phosphate and minerals are first translocated to the cotyledon from the endosperm and then transported to the developing part of the seeding axis (Organ *et al*., 1988). There is a correlation between the amount of phytin and the phytase activity of the seed. It has been showed that the amount of phytin in cotton seeds decreased quickly and disappeared after 6 days of germination. Meanwhile, the amount of inorganic phosphate and some other phosphorus containing compounds increased together with the phytase activity. In soybean seed germination, phytase activity increased maximal at 10 days after germination onset. Recently, phytase genes have been isolated from maize (*Zea mays*) and soybean cotyledons of germinating seeds (Maugenest *et al*., 1997).

In addition to phytase, germinating seeds contain various acid phosphatases which participate in the hydrolysis of phosphate-esters. Their activity also increases during germination (Biwas *et al*., 1991). Many APases have been identified in active form from various germinating seeds, such as four acid phosphatase forms in soybean seeds (*Glycine max*), six APase-forms in cotyledons of *Vigna mugo* seeds, and four Apase-forms in *Vigna sinensis* seeds (Haraguchi *et al*., 1990; Biwas *et al*., 1991). These APase-forms have specificities for various substrates such as phosphoenol pyruvate (PEP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), pyrophosphate (PPi), D-3-phosphoglycerate, D-glucose-6-phosphate or D-2,3-diphosphoglycerate. Biswas and Cundiff (1991) reported that acid phosphatase–III and IV of *Vigna sinensis* showed maximum activity with PEP and D-2, 3-diphosphoglycerate, whereas acid phosphatase-II has the highest activity with ADP. An APase was purified from castor bean seedlings (*Ricinus communis* L.) with 60 kD and maximum activity toward the tyrosine phosphate (Granjeiro *et al*., 1999).

Aims of the present study

Germination of seeds is a complex including the various functions of seed tissues and the different biochemical processes. These multi-stage processes are supposed to require the coordinated expression of various genes in the different tissues. During germination Pi stored in the endosperm is mobilized and transported to growing organs of seedlings, thus phosphate transporters and acid phosphatases are expected to play an important role in these processes. However, little is know about the function, mode of regulation and localization of phosphate transporters and acid phosphatases during this period. In order to get insight about the function and regulation of phosphate transporters and acid phosphatases during seed germination as well as in the development of *Ricinus* plants under phosphate starvation three topics were the aim of this study:

1. Determination of the translocation of Pi within the seedlings.

2. Isolation and characterization of phosphate transporter(s) and acid phosphatase(s) genes.

3. Subcellular localization of phosphate transporter and acid phosphatase transcripts by *in situ* hybridization.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

All chemicals which had been used in this work were obtained from Merck, Sigma, Roth, Ambion, Boehringer, Fermentas or Biochemica, unless otherwise stated in the text.
Radioactively labeled ^{32}P was obtained from Amersham Biosciences.

2.1.2 Enzymes and kits

T4 DNA ligase, Taq polymerase, DNA restriction enzyme were obtained either from MBI Fermentas (Vilnius, Lithuania) or New England Biolabs (Beverly, MA)
Calf intestinal alkaline phosphatase, RNase H (Promega GmbH)
T3 and T7 polymerase (Boehringer Mannheim GmbH)

Kits were purchased from:

First Choice RLM-RACE kit	Ambion, Texas, USA
Dig RNA labeling kit	Roche Biochemical, Mannheim, Germany
High Pure PCR product kit	Roche Biochemical, Mannheim, Germany
pMOSBLue blunt ended cloning kit	Amersham Biosciences
Agarose Gel DNA extraction kit	Roche Biochemical, Mannheim, Germany
Plasmid isolation kit	Nucleobond AX, Macherey-Nagel

2.1.3 Plasmid vectors

pGEM®-T easy for cloning of PCR product (Promega Madison)
pBluescript II KS for cloning of DNA templates for in vitro transcription (Stratagene)
pET28 for gene expression system in *E.coli* (Novagen)
p181A1NE for gene expression system in yeast *Saccharomyces cerevisiae*

pMOSBlue blunt ended cloning kit (Amersham Biosciences)

2.1.4 Plant materials and growth conditions

Castor bean seeds (*Ricinus communis* L.) were soaked in water overnight and were then surface-sterilized by 0.3% chinosol solution for 10 min. After three times washing in running tap water, the seeds were germinated on a petri plate containing 2% solidified agar in a dark chamber at 27°C for 2 days. Subsequently, germinated seeds were transferred into a hydroponics glass pot containing one-half-strength Hoagland solution (either 500 µM phosphate or no phosphate). The solution was renewed every 2 days and the growth of seedlings was continuously kept in a dark chamber at 27°C until 8 days.

For Pi starvation studies, 7 days old (after germination, DAG) plants were transferred to pots containing sterilized quartz sand in the green house and supplied daily with one-half-strength Hoagland's solution. Growing condition was 16-h-light/8-h-dark cycle (700-750 µmol photons m^{-2}). The harvested plant samples were stored at -80°C for further research.

The ½ -strength Hoagland's solution contained 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1.0 mM $MgSO_4$, 25 µM KCl, 23,1 µM H_3BO_3, 1.0 µM $MnSO_4$, 4.05 µM $ZnCl_2$, 0.19 µM $CuCl_2$, 0.05 µM Na_2MoO_4, 50 µM Fe-EDTA, 0.5 mM KH_2PO_4. The pH was adjusted to 5.8 using 0.1 M KOH. In case of nutrient solution without Pi, K_2SO_4 replaced KH_2PO_4 to maintain a constant K^+ concentration.

2.1.5 Bacterial strains

Table 2.1.1

Strain	genotype
DH5α	*Sup*E44 Δ*lacU*169(*Φ*80 *lacZ* ΔM15) *hsdR*17 *rec*A1 *end*A1 *gyr*A96 *thi*-1*rel*A1 (Sambrook et al., 1989)
BL21(DE3) pLysS	F^- ompT $hsdS_B$ (r_B^- m_B^-) gal dcm (DE3) pLysS (cam^R) (Novagen)

2.1.6 Yeast strains

Saccharomyces cerevisiae, yeast high-affinity phosphate transporter mutant NS219 (MPI, Golm, Germany)

2.1.7 Oligonucleotide Primers

Table 2.2.2: Sequence specific primers were used for amplification of full length clones, screening or incorporation of restriction sites. Degenerate primers were used for cloning of cDNA of *RcPT1* or *RcPS1*

A. Primers specific to sequence of vectors	
Name	sequence
T7	5´-TAA TAC GACTCACTATAGGGAGA-3´
T3	5´-AATTAACCCTCACTAAAGGGAGA-3´
S6	5´-ATTTAGGTGACACTATAGAAGNG-3´
B. Primers used for amplification of RcPT1	
PTRcF2 forward	5´-TCT CAC AAC CCA ACA AAC TC-3´
PTRcF4 forward	5´- TGG CTA CTA TAA AGA AGG TA-3´
PTRcR1 reverse	5´-AGA TGT AGT CTG CTT CTG GCA-3´
PTRcR2 reverse	5´-GAC ATG ATT GTT GCA GAG AG-3´
PTRcR3 reverse	5´-ATG GAT CTG AGA TTG CAC-3´
PT-RcF3 forward	5´-AAT CTT TAC CTA GCA GCA -3´
PT-RcR3 reverse	5´-CAG CAC AGA GAG TGA TAA ACT-3´
Degenerated primers	
PTRcF1 forward	5´-ATG GGW TTY TTY ACW GAT GC-3´ (W=A/T;Y=C/T)
PTRcR1 reverse	5´-CCD AAA TTW GCD AAD AAD AAW-3´ GT (W= A/T; D=A/G/T)
C. Primers used for amplification of RcPS1	
APRcF4 forward	5´-CT TTG CCC ACC AAA CAT GTG CAA G-3´
APRcF5 forward	5´-AGC AAT CCT ATG GTT ATC AAG GCA G-3´

APRcR2 reverse 5′-GGG CAG AAA TCA CCA ATT CCA TC-3′

APRcR3 reverse 5′-CTT GCA CAT GTT TGG TGG GCA AAG-3′

AP-RcF1 sense * 5′-GGA GAT TCC ATA TGAATGGCTGGAATTTTGGTTGTT-3′

AP-RcR1 reverse ** 5′-CGC GGA TCC GGC AAG GCA GCA ATT GGT ACA G-3′

* containing *Nde I* site underline; ** containing *BamHI* site underline

Degenerated primer

APRcF1 forward 5′-GTD TTY GAT TTY GAT AAD ACW ATY AT-3′ (W=A/T; Y=C/T) (W= A/T; D=A/G/T)

APRcF2 forward 5′-GAT GCW AAY GTD TTY TTY ATY GA-3′ (W=A/T; Y=C/T; D=A/G/T)

APRcR1 reverse 5′-GGD CAD AAA TCW CCA ATW CCA T-3′ (W=A/T; D=A/G/T)

D. RLM-RACE primers	
5′RACE adapter	5′- GCUGAUGGCGAUGAAUGAACACUGUUGCUGGCUUUGAUGAAA-3′
3′RACE adapter	5′- GCGAGCACAGAATTAATACGACTCACTATAGGT$_{12}$VN-3
5′Outer primer	5′-GCT GAT GGC GAT GAA TGA ACA CTG
5′Inner primer	5′-CGC GGA TC GAA CAC TGC GTT TGC TGG CTT TGA TG
3′Outer primer	5′-GCG AGC ACA GAA TTA ATA CGA CT
3′Inner primer	5′-CGC GGA TCC GAA TTA ATA CGA CTC ACT ATA GG

2.1.8 Bioinformatics tools and computer analysis

Chromas for sequence reading

GCG for various sequence analysis

Sequencing analysis was carried out with the University of Winconsin GCC package (Version 10.3, Genetics Computer Group (GCC), Madison, USA). Amino acid sequences of putative high-affinity phosphate (Pi) transporter or acid phosphatase were aligned by using ClustalX (Thomson *et al*., 1997).

Blast for finding similar database sequences http://www. ncbi.nlm.nih.gov/Blast

Entrez Pubmed for sequence retrieval and data http://www. ncbi.nlm.nih.gov/Entrez

MAP for multiple sequence alignments http://www.genome.cs.mtu.edu/map.html

SigmaPlot (SPSS, Chicago, USA) program for statistical analysis.

2.2 Methods

2.2.1 Determination of Phosphorus

The optical Simultane-Sequenz-Spectrometer with inductively coupled Argon-plasma enables the simultaneous determination of up to 27 elements in aqueous solutions. The sample solution is nebulized and transported by an argon stream into the plasma where it is vaporized, atomized, partially ionized and induced to optic emission. Spectral analysis of the emitted light is done by a polychromator or by a Czerny-Turner Monochromator and the intensity of spectral lines is measured by photomultipliers. Plant tissue samples were dried at approximately 70^{o}C in a forced air oven for 2 days. All tissue samples were ground to pass a 2 mm mesh sieve and digested for 16 h at 65°C with 2 ml concentrated HNO_3. Ion content of the samples was determined by ICP-AES (inductively coupled plasma atomic emission spectrometer).

2.2.2 Application of radiotracers

Radioisotope ^{32}P (Amersham Bioscience, Freiburg, Germany) was used as a tracer for phosphorus. ^{32}P was added into the solution with an inactive KH_2PO_4 / K_2HPO_4 buffer, pH 7.5, to a final concentration of 2 mM or 10 mM phosphate. The tracer was applied either to cotyledons or roots of 7 days old (DAG) seedlings.

a) Incubation of the cotyledons

The endosperm of seedlings was removed and the cotyledons were placed in the incubation buffer (5 mM 2(N-morpholino) ethanesulfonic acid (Mes)-KOH buffer, pH 5.5; 0.2 mM $CaCl_2$, 100 mM sucrose). Phosphorus tracer was added to phosphorus-free solutions. The seedlings were fed with a radioactive tracer via the cotyledons, subsequently the seedlings were carefully wrapped in plastic foil and shock-frozen at -80°C for further analysis.

b) Incubation of the roots

The endosperm of the seedlings was removed and then the seedlings were placed into the incubation buffer (1 mM 2(N-morpholino) ethanesulfonic acid (Mes)-KOH buffer, pH 5.5;, 0.5 mM $CaCl_2$ and either 2 mM or 10 mM phosphate buffer (KH_2PO_4/K_2HPO_4, pH 7.5). The seedlings were fed with a radioactive tracer via the roots for 1 hour,

subsequently seedlings were carefully wrapped in plastic foil and shock-frozen at -80°C for further analysis.
A pre-incubation time of 7 hours was chosen to allow an equilibration of the fluxes for both cases (a) and (b). After harvest, each seedling was separated into cotyledons, hypocotyl and roots, and these were separately assayed for radioactivity.

2.2.3 Autoradiography and tracer detection

The distribution of radiotracer within leaves was monitored by phospho-imaging technique (Fuji BAS 1800, FUJI PHOTO FILM, Japan). Leaves or plants were exposed for 10 - 30 minutes to a sensitive imaging plate, which was then scanned with a laser beam and the image data were read to a computer. This method offered a facility to obtain very quickly isotope distribution patterns in fresh material.
Shock-frozen seedlings were exposed to the imaging plate at -80°C in order to avoid thawing of the sample and redistribution of tracer. The imaging plate was pre-cooled to -80°C within a metal exposure cassette for about 30 min. After exposure, samples were left at -80°C for a subsequent analysis of tissue samples. The imaging plate was brought to room temperature and then scanned. For this procedure, the imaging plate was wrapped in transparent plastic film to minimize contact with water, especially condensation water. Exposure time was not prolonged compared to room temperature. With digitalized autoradiographic pictures, an image analysis could be performed (AIDA image analyser, RAYTEST, Straubenhardt, Germany), which gave information on activity per area or activity profiles. Activity was calculated in comparison to a calibration standard exposed simultaneously. Drying or thawing of seedlings before autoradiography was always avoided, as it may lead to substantial redistribution of tracer within the drying plant (Barrier and Loomis, 1957).

Alternatively, to verify quantitative results from image analysis, activity in tissue samples was detected by scintillation counting. Samples of cotyledons or hypocotyl tissues (usually 20 - 100 mg) where excised from the seedling and weighed. They where solubilised in organic base (Soluene 350, CANBERRA PACKARD, Dreieich, Germany) overnight. The supernatant was then added to scintillation liquid (Hionic Fluor, CANBERRA PACKARD) and the activity was determined by a scintillation counter (Beckman LS 1701) in relation to previously prepared standards. The transported

amount of ^{32}Pi was calculated under the assumption that the ratio between active and inactive P is the same as in the feeding solution.

2.2.4 Isolation of DNA and Southern Blot analysis

Genomic DNA was isolated from *Ricinus* plant. Fresh tissue was frozen in liquid nitrogen before extraction and ground to a fine powder. The powder tissue was placed in a 3 vol. of extraction buffer (0.1 M Tris-HCl pH 8.0, 0.5 M NaCl, 50 mM EDTA, 1.5% CTAB, 1.5% β-mercaptoethanol (added just before use), 2% SDS. After mixing gently, the mixture was kept for 20 minutes at 65°C. Then 5 M potassium acetate was added to this suspension to a final concentration of 0.5 M and precipitated with 0.6 vol. isopropanol on ice. The pellet was dissolved and purified by phenol-chloroform-isoamylalcohol extraction. Dry pellet was dissolved with water and DNA concentration was measured by photometer. Aliquots of 10µg of total DNA were used for digestion with several restriction enzymes. The digest DNA samples were separated on a 0.8% agarose gel in 0.5x Trisborate-EDTA (TBE) buffer (pH 8.0) and blotted in Hybond N+ nylon membrane by capillary transfer with 20x SSC, UV crossed linked and hybridized with specific Dig-RNA labelled at 55°C in buffer containing 50% (v/v) formamide, 5x SSC, 0.1% (w/v) sarcosyl, 0.02% (w/v) SDS, and 1% (w/v) BMB blocking reagent. After hybridization, membranes were washed once in 2x SSC, 0.1% SDS (for 15 min at room temperature); twice in 0.1% SSC, 0.1% SDS (for 15 min at 55°C) and then exposed to Kodak films.

2.2.5 Isolation of RNA and Northern Blot analysis

Frozen tissue (0.5-1 g) was ground in liquid nitrogen to a fine powder, then homogenized with 2 volumes of guanidine buffer (8 M guanidine hydrochloride, 20 mM EDTA, and 50 mM β-mercaptoethanol at pH 7.0). The guanidine hydrochloride extract was centrifuged in 10.000 rpm for 10 min at 4°C. Then, supernatant containing RNA was extracted one time with phenol-chloroform-isoamyl alcohol and one time with chloroform. The aqueous phase was mixed with 2 volumes of ethanol and 0.3 M of sodium acetate and kept at -20° overnight. The pellet after centrifugation at 10.000 g was resuspended in 2 M LiCl, 10 mM sodium acetate pH 5.2 for 1 hour at 4°C and centrifuged again. The RNA pellet was washed with ethanol and then dissolved in water. Ten µg RNA were used for

Northern blot. RNA samples were denatured and run on 1.2% agarose gel containing formaldehyde, then blotted to Hybond N+ nylon membrane. Hybridization and detection procedure are the same as for Southern blot (2.2.4).

2.2.6 cDNA synthesis by reverse transcriptase polymerase chain reaction (RT-PCR)

A first strand cDNA was synthesized from mRNA of *Ricinus* cotyledon by using Superscript II RT enzyme (Invitrogen), which primes to the 3´ poly-A tail of the mRNA.

Reagents:
Oligo $(dT)_{12-18}$ primer
Superscript II RT enzyme
5x first strand buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)
DTT 0.1mM
dNTP 10mM
DEPC water.
The synthesis reaction was carried out as the manufacture's instruction: 1µl Oligo $(dT)_{12-18}$ primer was added into 10 µl RNA (1µg) and incubated for 15 min at 65°C and cooled down on ice. Then the reaction was mixed with 1µl dNTP, 1 µl RNase inhibitor (1 U), 2 µl DTT, 4 µl first strand buffer, 1 µl Superscript II RT enzyme (200 U) and incubated for 60 min at 42°C. The reaction was stopped by heating at 95° for 5 min.

2.2.7 Polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) is a powerful tool for molecular biology research. The PCR product was amplified by exponential process since amplified products from the previous cycles serve as templates for the next cycle of amplification.

2.2.7.1 Standard PCR

A standard PCR was used for several applications during this work such as: screening of transformed bacterial clones and amplification of DNA fragments from plasmid or genomic DNA. The components of a standard PCR protocol using Taq DNA polymerase are as follows:

DNA template	2-5 µl
5´-prime (10 pmol/ µl)	1 µl
3´-prime (10 pmol/ µl)	1 µl
10x PCR buffer (100 mM KCl, 100 mM	1 µl
Tris-HCl pH 8.0, 1.5 mM $MgSO_{4)}$	1 µl
dNTP (10 mM of each nucleotide)	1 µl
Taq Polymerase (2U/µl)	1 µl
Add H_2O	50 µl.

Three distinct cycles were used for this standard PCR: 1) 60 sec at 94°C to denature DNA; 2) 25-30 cycles of: 30 sec template denaturation at 94°C, then 30 sec annealing of primers to DNA template (depending on the homology of primers for the target sequence as well as the base composition of the oligonucleotides), and then elongation time depending on the length of expected product (*Taq* Polymerase at 72°C); 3) 10 min extension of finished products at 72°C.
The PCR reactions were performed in DNA Thermal Cycler (PerkinElmer, Life Science, Rodgau-Jügesheim).

2.2.7.2 Screening bacterial colonies with PCR

For screening transformed bacterial colonies, suspected positive colonies were resuspended in 5 µl H_20, 2 µl solutions from this mixture was used for standard PCR (see 2.2.7.1) with specific primer of insert and primer specific for the vector. 3 min at 94°C is required to break the cells before starting the PCR process.

After the PCR products were examined on agarose gel, 3 µl of colonies were mixed in H_2O and transferred into culture tubes with LB medium containing appropriate antibiotic. The cultures were grown overnight at 37°C, 250 rpm shaking followed the isolation of plasmid DNA.

2.2.7.3 Design of specific and degenerate primers

In PCR primer design is one of the most important factors. The amplification product of a PCR reaction is defined by the sequence of the PCR primers. The ideal primers only anneal to complementary sequences of target DNA and not to other sequences in the sample reaction. Following features are needed for design of specific primers: Primers are 18 to 24

nucleotides in length. Select-primers contain 40% to 60% GC or mirror the GC content of the template. Design-primers contain G or C residues in the 5´ or central regions to avoid complementary sequences at the 3´ end of primer pairs. Primer sequences should avoid the potential to form internal secondary structure. The melting temperature (T_m) of the primer should not be higher than the amplification temperature. Annealing temperatures are generally set about 5°C below the T_m of the primers. Degenerate primers for cloning a cDNA of a phosphate transporter or an acid phosphatase were designed based on an alignment of known amino acid sequences from other plants (Table 2.2.2 B, C).

2.2.7.4 Cloning of amplified products

PCR products were excised from agarose gel by purification with help of a DNA kit from agarose gel and ligated into either pGEM®-T easy or pMOSBlue vectors. These both vectors contain the genetic marker lacZ reporter gene, which enables to screen host cells containing vectors by blue-white selection.

2.2.8 RNA Ligase Mediated Rapid amplification of cDNA Ends (RLM-RACE)

The advantage of RLM-RACE over regular RACE technique (Maruyama.K *et al*., 1994; Shaefer, 1995) is that it can solve two common problems which generate truncated cDNAs: 1) Reverse Transcriptase often does not copy the mRNA through secondary structure to its 5' end and 2) PCR preferentially amplifies shorter fragments selecting for incomplete cDNA's. To accomplish amplification of cDNA's which go to the very end, a transcribed RNA linker is ligated only to those messages that have had a 5' methyl cap. For this purpose, an enzyme, TAP, is used that specifically targets the unique linkage at the 5' methyl cap. On capped messages, TAP generates a phosphoryl group (a donor for ligation). Since the decapping is done only after CIP destroyed phosphates at the end of degraded messages, only the full length message can act as a substrate in the ligation. Moreover, due to reverse transcription after the ligation, only reverse transcriptions that proceed through the entire message will have a linker, thus only full length cDNAs will have primer binding sites for PCR.

2.2.8.1 5´ RACE

The amount of RNA for one reaction was 10 µg total RNA or 250 ng poly-(A) RNA, 2 µl 10x CIP buffer, 2 µl Calf Intestinal Phosphatase (CIP) and H_2O added to total volume of

20 µl. The procedure was: mix gently, spin briefly and incubate at 37°C for one hour. then terminate the CIP reaction and extract the mixture with 15 µl ammonium acetate, 115 µl H_20, 150 µl acid phenols: chloroform, and finally vortex briefly and centrifuge for 5 min at room temperature. After washing one time with chloroform, the aqueous phase was precipitated with 150 µl isopropanol. The pellet was rinsed with 0.5 ml 70% ethanol and allowed to dry at air. 10 µl H_20 was used to dissolve the pellet, then mixed gently with 5 µl of solution containing 1 µl 10x TAP buffer, 2 µl Tobacco Acid Pyrophosphatase (TAP) and 2 µl H_2O. Incubated for one hour at 37°C, then 2 µl CIP/TAP treated RNA was mixed with 1 µl 5´RACE adapter, 1 µl 10x RNA ligase buffer, 2 µl T4 RNA ligase (2.5 U/µl), 4 µl H_20 and incubated again for one hour at 37°C. The Reverse Transcription steps were done, except when random decamer primers were used instead of Oligo (dT) $_{12\text{-}18}$ primer. One µl cDNA product was used as a template for a Nested PCR, the other components of the reaction mixture were added together with 5´ RACE outer primer and specific primer *PTRcR1* or *APRcR2* (Table 2.2). The PCR cycle was as follows: 3 min at 94°C; 35 cycles: 94°C 30 sec, 60°C 30 sec, 72°C 30 sec; 7 min at 72°C. Second Nested PCR was performed with the previous PCR product, 5´RACE Inner primer and either gene-specific primer *PTRcR2* or *APRcR3* in the same condition as at the first PCR reaction. Finally, the PCR product was cloned in pGEM®-T easy and sequenced.

2.2.8.2 3´ RACE

For reverse transcription, 2 µl (1 µg total RNA), 4 µl dNTPs (10 mM) and 1 µl RNase inhibitor (20U), 2 µl 3´RACE adapter, 4 µl 5 x RT buffer, 1 µl Superscript II RT enzyme and 6 µl dd H_2O were used. The mixture was incubated at 42°C for 1 h and then applied to PCR. The PCR component is the same as that mentioned in 2.2.6.1, except 1 µl cDNA was used for a PCR with the 3´RACE gene-specific outer primer (Table 2.2D) and inner primers were used with specific primers *PTRcF2* or *APRCF4*. PCR cycle as follows: 3 min at 94°C; 35 cycles: 94°C 30 sec, 60°C 30 sec, 72°C 30 sec; 7 min at 72°C. The PCR product was cloned in pGEM®-T easy and sequenced.

2.2.9 Nonradioactive RNA probe synthesis

RNA probes of Pi transporter, *RcPT1* and acid phosphatase, *RcPS1* were produced by using PCR generated templates for in vitro transcription. Briefly, the DNA fragments

generated by PCR with gene-specific primers were cloned into pGEM®-T Easy. The orientation of the insert fragment was determined by sequencing or by PCR with known primers which were located within the fragment and the vector, such as T7 or SP6 primers.

For the transcription of RNA sense or anti-sense probes, the vectors were linearized with an appropriate restriction enzyme that creates 5´ overhangs. This step helps to avoid transcription of undesirable sequences. After digestion, DNA was purified by phenol/chloroform extraction, followed by ethanol precipitation. The transcription reaction was performed essentially according to the instructions of manufacturer (Roche Biochemical, Mannheim, Germany). 1 µg of linearized plasmid DNA was used to synthesize RNA probes. The reaction mixture contains:

1 µg linearized plasmid DNA dissolved in 13 µl double distilled water (dd H_2O)
2 µl 10 times concentrated Dig RNA labeling mix (10 mM each of ATP, CTP and GTP, 6.5 mM UTP, 3.5 mM Dig-11-UTP, pH7.5)
2 µl 10 times transcription buffer (400 mM Tris-HCl, pH 8.0, 60 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 20 mM spermidine)
2 µl RNA polymerase either SP6 or T7
1 µl RNase inhibitor.

After 2 h incubation at 37°C, 2 µl DNase I was added to remove template DNA and incubated for 15 min at 37°C. The reaction was stopped by adding 2 µl 0.2 M EDTA, pH 8.0. Labeled probes were purified by using High purification Kit (1.1.2) to remove template DNA. Finally, the RNA probe was precipitated by LiCl/ethanol, 2.5 µl 4 M LiCl and 75 µl cold 100% ethanol mixed with 20 µl RNA probe solution that was obtained from the purification step above, and incubated for 3 h at 20°C. The RNA probes were precipitated by centrifugation (at 13.000 rpm) for 15 min at 4°C. The supernatant was removed and the pellet washed with cold 70% ethanol to remove the remaining free nucleotides. The RNA probes were dissolved in RNase free double distilled water and stored at -20°C.

2.2.10 *In situ* hybridization

2.2.10.1 Fixation and embedding of sample

Plant tissues of *Ricinus* were cut into small pieces and transferred to glass vials containing fixative solution (50% ethanol, 5.0% acetic acid, 3.7% formaldehyde). Tissues with fixative were placed in a vacuum (obtained with an aspirator) for 15 minutes. The vacuum was pulled very slowly in order to pull the air out of the tissue, allowing the fixative to penetrate. After 15 minutes of slow bubbling, the vacuum was released slowly, and then samples were incubated at room temperature for 2-4 hours. The fixative was removed and a series of ethanol steps were started after washing two times with 1x phosphate buffer (0.13 M NaCl, 0.7 mM Na_2HPO4, 0.3 mM NaH_2PO_4) and continued 60 min each of 30% graded ethanol (EtOH), 50% EtOH, 70% EtOH, 80% EtOH, 90% EtOH and 95% EtOH with 0.1% Eosin (Sigma) overnight. Ethanol-eosin was replaced by 100% EtOH and incubated twice for 1 hour each. Tissues were permeated with Histoclear (Roth, Karlsruhe) before embedding with paraffin, 30 min in 25% Histoclear-75% EtOH, 30 min 50% Histoclear-50% EtOH, 30 min 75% Histoclear-25% EtOH, two times 60 min each in 100% Histoclear and finally 100% Histoclear with ¼ volume paraplast chips (Para X-tra, Sigma) overnight at room temperature. Next day, vials containing paraplast were placed in 42°C after the chips were dissolved, ¼ volume of paraplast chip was added and vials were moved to 56°C for several hours. Fresh melting paraplast was replaced for 6 changes over 3 days. The samples were embedded in plastic mold and stored at 4°C. Eight µm thick sections were cut with disposable knife on a Microtom (2050, Reichert-Jung, Leica Microsystem, Nussloch) and placed on pre-cleaned coated glass slides (Superfrost®Plus, Menzel-Gläser®, Brauschweig) followed by incubation for 24 hours at 42°C. The slides were stored at 4°C.

2.2.10.2 Pre-hybridization and hybridization

Slides were incubated two times in Histoclear for 10 min each to remove paraffin and 2 times in 100% EtOH for 1 min each followed by a stepwise series of redehydration with 95% EtOH, 90% EtOH, 80% EtOH, 60% EtOH, and 30% EtOH for 1 min each. Then the slides were washed two times in ddH_2O for 1 min each, followed by one time washing 2X SSC at room temperature. Proteinase K treatment was used to partially digest the tissue for 10 min at 37°C. The sections were re-fixed for 10 min in fresh 4% paraformaldehyde. Positive charges in tissues were removed by 0.1 M triethanolamine with acetic anhydride

pH 8.0. Pre-hybridization solution (50% deionized formamide, 10% (w/v) dextran sulfate, 1x Denhardt´s solution, 1 mg/ml tRNA, 1 mg/ml DNA salmon sperm in 0.6 M NaCl; 10 mM Tris-HCl, pH7.4; 1 mM EDTA, pH8.0) without probe was applied to each slide and distributed all over the tissues. Following incubation in pre-hybridization buffer at 45°C for 2 h, the sections were incubated with hybridization solution which contained approximately 0.5 µg/ml DIG-labeled probes in pre-hybridization buffer. On each slide 100 µl hybridization solution were applied. Slides were then incubated in a humidified chamber at 42°C for 16 h.

After hybridization, slides were sequentially washed with 50% (v/v) formamide/2 X SSC at 42°C (20 min X 3 times) and NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) at 37°C for 5 min. Then sections were treated with RNase A, 20 µg/ml, in NTE buffer at 37°C for 30 min, followed by washing with NTE buffer at 37°C for 5 min, and with 0.1 X SSC at 42°C three times for 20 min.

After washing with buffer 1 (0.1 M Tris, 0.15 M NaCl, pH 7.5) at room temperature for 1 min, the slides were incubated with 5 % (w/v) blocking reagent (Boehringer Mannheim GmbH, Germany) in buffer 1 at room temperature for 30 min. Then the slides were incubated in a humidified chamber overnight at 4°C with anti-DIG antibody (Boehringer Mannheim GmbH, Germany), diluted to 1:100 in buffer 1 containing 1% blocking reagent; and then 200 µl antibody solution were added to each slide. Negative controls were conducted by addition of sense probe instead of anti-sense probe or by omission of anti-sense probes in the hybridization solution. Slides were then washed with buffer 1 two times for 15 min each and then PBS for 5 min at room temperature. Afterwards slides were equilibrated for 5 min each in buffer 2 (100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM MgCl,) and then incubated overnight up to several days in the dark at room temperature with buffer 3 containing 4.5µl/ml NBT and 3.5 µl/ml BCIP (150 µl/slide). The signal was checked frequently. When the color on the slide was optimal, the color development was stopped by washing the slide three times for 5 min each in distilled water. Before analysis under the microscope, the slides were dehydrated by incubation in 70% EtOH for 15 seconds and then in 100% EtOH two times for 15 seconds each. The sections were observed under a light microscope (BH2, Olympus, Hamburg) with a digital camera (DXC-101P, Sony®) and computer software Image-Pro Plus 3.01 (Media Cybernetics®, Gleichen).

2.2.11 Expression of *RcPT1* in yeast mutants

2.2.11.1 Transformation of yeast

The cDNA of putative phosphate transporter *RcPT1* was subcloned into yeast expression vector p181A1NE and transformed into yeast phosphate uptake mutant MB 192 by following method.

Reagents:
10x LiAc: 1 M LiOAc (adjusted to pH 7.5 with dilute acetic acid)
10x TE: 0.1 M Tris-Cl (pH 7.5),
10 mM EDTA
50% PEG 4000 in water (autoclave ONCE only)
PEG/LiAc solution: 40% PEG 4000, 1X TE, 1X LiOAc
LiOAc solution: 1 ml 10x LiOAc, 1 ml 10x TE, 8 ml H_2O.

Five ml YPD medium were inoculated with a single yeast colony and grown over night at 30°C. Then 0.5 - 4.5 ml culture was added into 50ml fresh media YPD, checked until a cell density of 0.2 (2 x 106 cells/ml) at 600 nm. The culture was incubated at 30°C on shaker (200 rpm) until 1.0 (2 x 107 cells/ml) at 600 nm was reached which took approximately 3-5 hours. 50 ml were spun down cells at 5000 rpm for 5 minutes and then the medium poured off. The pellet was resuspended in 10 ml sterile water, then centrifuged for 5 minutes, the water poured off and the pellet resuspended in 1 ml 1X LiOAc. The suspension was transferred to a 1.5 ml sterile microfuge tube, spun down at top speed for 15 sec and the LiOAc solution removed completely. The pellet was resuspended in 250 µl LiOAc (4x109 cells/ml). 50 µl yeast cells were mixed with transforming pDNA (1-5 µg) and 5 µl single-stranded carrier DNA (10 mg/ml, boiled 10 min at 95°C and quick chilled on ice, 10 min) in a 1.5 ml microfuge tube. 300 µl sterile PEG/LiAc solution were added and thoroughly mixed and incubated at 30°C for 30 minutes with occasional gentle shaking. Yeast cells were treated under heat shock at 42°C for 15 minutes. The cells were immediately chilled on ice and centrifuge 5 seconds at 6-8000 rpm and then the supernatant was removed. The yeast cells were resuspended in 1 ml 1X TE (pipetting it up and down gently at this step to obtain high efficiency; excessive washing may wash away transformants). 200 µl of transformation mix were plated on selective media plate and incubated at 30°C for 2-3 days.

2.2.11.2 Acid phosphatase activity test and yeast growth experiments

Yeast cells were plated on YPAD medium in which sucrose was replaced by glycerol (30 g/l) and were stained for repressible acid phosphatase activity by embedding the colonies in the agarose containing components of a diazo-coupling reaction mix (Bunya *et al*., 1991). The mixture consisted of naphthylphosphate and o-dianisidine dissolved in 0.1 M acetate buffer, pH 4.0. Red dye precipitation indicated the presence of phosphatase activity. Cell growth was monitored in a liquid YPAD low phosphate medium by measuring the optical density at 600 nm.

2.2.11.3 Uptake of ^{32}P in yeast

The yeast cells were grown to the logarithmic phase on YNB medium (Difco, Chemie Brunschwig AG, Basel, Switzerland) containing 140 µM KH_2PO_4, then the cells were washed in Pi-free medium and resuspended in the same medium and incubated at 30 °C for 4 hours. For the growth experiments, cells were cultured for 35 hours in YNB medium. For uptake experiments, cells were washed and resuspended and then activated in 20% glucose to optimize energization of the plasma membrane. 20 µl cell suspension were incubated at 30 °C in the presence of 14.8 kBq ^{32}P for up to 5 min. Uptake was stopped by addition of 10 ml ice-cold water and then filtered by glass fiber filter. The radioactivity incorporated by the cells was determined by using a liquid scintillation counter.

2.2.12 Expression of RcPS1 recombinant protein in *E.coli* using pET system

The full length *RcPS1* cDNA was amplified by PCR with a primer pair AP-RcF1 and AP-RcR1 containing restriction sites *NdeI* and *BamHI* (see table 1) and subcloned in frame into *NdeI* and *BamHI* sites of the pET28 (+) vector (Novagen). This construct was then verified by sequencing. Recombinant RcPS1-pET28 (+) was transformed into *E.coli* BL21 (DE) cells. A single positive colony was grown over night at 37°C in 10 ml LB medium containing 100 µg/ ml ampicillin. Five ml overnight culture were transferred into 100 ml LB containing 2% glucose and 100 µg/ ml ampicillin and grown at 37°C until reached ~ 0,5 units at 600nm. IPTG was added into culture to a final concentration of 1 mM. After 3 hours induction at 37°C, cells were centrifuged 5,000xg for 5 min at 4°C.

2.2.13 Protein analysis

2.2.13.1 Purification of recombinant protein

Cells were pelleted and well resuspended in lysis buffer (25 mM Tris-HCl pH 8.3, 2 mM EDTA, 50 mM NaCl, 1 mM PMSF) then lysozyme at 100 µg/ml was added and incubated for 20 min at room temperature until the suspension became turbid and viscous. The cell suspension was sonicated with 10 short burst of 10 sec followed by intervals of 20 sec on ice. Cell debris was removed by centrifugation at 40,000 g for 20 min at 4°C. Fusion protein was purified by Ni-NTA agarose. Protein purification step was performed following the protocol provided by manufacturer (Novagen).

2.2.13.2 Protein extraction from plants

Plant samples (10 g) were ground in 15 ml ice cold buffer (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, 0.5 M sucrose, 100 µM PMSF). The homogenate was filtered through 4 layers cheesecloth followed by centrifugation at 8,000g for 10 min at 4°C to remove cell debris, plastids, and starch. The supernatant was transferred to ultracentrifuge tubes and centrifuged at 100,000 g for 60 min. The pellet was resuspended in 500 µl resuspension buffer (100 mM HEPES (pH 7.0), 0.05% Triton X-100, 0.5 M sucrose) and pelleted at 8,000 g for 10 min at 4°C. The protein concentration was measured according to Bradford (1976).

2.2.13.3 SDS-PAGE

Proteins were separated by gel electrophoresis on polyacrylamide gel. Glass plates, comb and plastic spacer were cleaned and assembled. Separating gel solution of 10% acrylamide was prepared and poured into the gap between the glass plates. One cm space from the gel surface to the ends of teeth of the comb was left. Two ml of isopropanol was added to cover gel surface, this prevents oxygen from diffusing into the gel and inhibiting polymerization. After 30 min, polymerization was completed. Isopropanol was removed and gel was washed 5 times with ddH_20. Before adding stacking gel, all remaining water was removed, and then the comb was placed between glass plates. While polymerization proceeded, protein samples were boiled in SDS sample buffer for approximately 5 min and loaded into the slots. Gel electrophoresis was run at 15 mAmp constant current until the

Bromophenol Blue dye front moved through the stacking gel followed by set 30 mAmp constant current until the dye moved to the bottom of the gel.

1X SDS sample buffer: 0.1% (w/v) Bromophenol Blue, 2% (w/v) SDS, 10% (v/v) glycerol, 100 mM DTT, 50 mM Tris-HCl (pH 6.8).
Electrophoresis buffer: 0.025M Tris, 0.1% (w/v) SDS, 0.25 M glycine.
Separating gel: 0.125 M Tris-HCl, 0.1% SDS, 7.5% (w/v) acrylamide, 0.2% (w/v) N, N-methylenbisacrylamide, 0.05% (w/v) ammonium persulfat, 0.1% (v/v) TEMED.
Stacking gel: 0.125M Tris-HCl, 0.1% SDS, 4% (w/v) acrylamide, 0.11% (w/v) N,N-methylenbisacrylamide, 0.05% (w/v) ammonium persulfat, 0.1% (v/v), TEMED.

2.2.13.4 Coomassie staining of protein gel

The gel was immersed in Coomassie stain solution (0.18% Coomassie Brilliant Blue R-250, 25% methanol, 10% glacial acetic acid) with gent shaking until the protein of interest became visible. Then the gel was distained in Coomassie stop solution (25% methanol, 10% (v/v) glacial acetic acid) overnight. The gel was then rinsed with ddH_2O before going to Western blot or vacuum drying.

2.2.13.5 Antibody Production

A peptide corresponding to the carboxyl terminus of RcPT1 protein (NH_2-CNEDEPQEEAISSRT-$CONH_2$) was synthesized by Pineda (Pineda Antikörper-Service, Berlin, Germany). This synthetic peptide was used as the antigen for preparation of RcPT1-specific antibodies. An additional cysteine residue was added to the N-terminus of the peptide for coupling to keyhole limpet hemocyanin (KLH) for antibody production. The antibodies were prepared by Pineda.

The antibodies derived from the injection of synthetic peptide were affinity purified as follows. In preparing the gel step, 0.1 g CNBr-activated Sepharose 4B (Amersham Biosciences, Freiburg) was suspended in 1 mM HCl buffer. When the gel was swollen, it was washed twice, 15 min for each, with 1 mM HCl buffer on a sintered glass filter. Two milligram peptide were dissolved in coupling buffer 0.1 M $NaHCO_3$ (pH 8.3), containing 0.5 M NaCl and then mixed with preswollen Sepharose 4B gel. After two hours rotation at room temperature, the mixture was washed with coupling buffer of at least 5 times of the gel volume to remove excess ligand. Any remaining active groups were blocked with 0.1 M Tris-HCl buffer (pH 8.0) for 16 hours at 4°C. The medium was washed three cycles of

alternating pH, each cycle consisting of a wash with 0.1 M acetate buffer NaOAc (pH 4.0), 0.5 M NaCl and subsequently a wash with 0.1 M Tris-HCl (pH 8.0), 0.5 M NaCl. After a washing step with phosphate buffer saline (PBS) and an equilibration with 0.5 M NaCl, the antibody was eluted by adding 0.1 M glycine-HCl (pH 2.4). Collected antibody was added to 1% serum albumin (BSA) for long term store.

2.2.13.6 Western blot

Polyacrylamide gel was briefly rinsed in Transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.01% SDS, 10% methanol) and assembled with PVDF membrane (Immobilon P, Millipore, München) in between of wetted Whatman 3MM paper. Protein from gel was transferred into PDVF membrane at a constant voltage of 50 V for 4 to 5 hours. The membranes were blocked in 3% gelatine in TBS (20 mM Tris-HCl/ 0.5 M NaCl, pH 7.5) for 30 min at room temperature and then washed twice with TTBS (1xTBS+ 1% Tween-20) for 5 min each. The membranes were incubated with 1:1000 dilution of RcPT1 antibodies in TTBS containing 1% gelatine for 2 hours and then washed three times with TTBS followed 1 hour incubation with secondary antibodies at 1:2000 dilution. After washing two times with TTBS and one time with alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50mM $MgCl_2$) the membranes were incubated in BCIP/NBT substrate for colour development.

2.2.13.7 Immunolocalization

The samples were prepared as mentioned in section 2.2.10.1. Paraffin was removed from the slide (2.2.10.2). The slides were re-hydrated through a series of decreasing ethanol, then incubated with 0.5% Triton X-100 for 10 min at room temperature. After washing two times with wash buffer (1x PBS, 0.1% Tween 20, 20 mM glycine, 1% BSA) for 30 min at room temperature, the BSA was removed and the slides were incubated with RcPT1 antibody (1:500) for overnight in PBS containing 0.5% (w/v) BSA at room temperature followed by washing three times with wash buffer for 10 min each at room temperature. 1:2000 dilution of secondary antibody conjugate was applied to each slide. The slides were incubated for 1 hour at room temperature and washed two times with wash buffer. Afterwards the slides were equilibrated for 5 min each in buffer 2 (100 mM Tris-HCl pH 9.5; 100 mM NaCl; 50 mM MgCl,) and then incubated overnight up to several days in the dark at room temperature with buffer 3 containing 4.5µl/ml NBT, 3.5 µl/ml BCIP (150 µl/slide). The signal was checked frequently. When the color on the slide was optimal, the color was

stopped by washing the slide three times for 5 min each in distilled water. Before analysis under the microscope, the slides were dehydrated by incubation in 70% EtOH for 15 seconds and then in 100% EtOH two times for 15 seconds each. The sections were observed under a light microscope (BH2, Olympus, Hamburg) with a digital camera (DXC-101P, Sony®) and computer software Image-Pro Plus 3.01 (Media Cybernetics®, Gleichen).

3 Results

3.1 Characterization of Pi translocation in *Ricinus communis* L. seedlings

Phosphate is an important nutrient for plants. The movement of Pi is considered as highly mobile within plants. It is transported in xylem as well as in phloem in appreciable amounts and may also be exchanged between these two pathways (Mimura, 1995). The main purpose of these experiments is to identify the distribution and translocation of Pi within the seedlings via phloem and via xylem.

3.1.1 Translocation of phosphate from the cotyledons to the hypocotyls

The translocation of Pi from the cotyledons to hypocotyls was investigated by using (^{32}P) as a tracer. As it shows in figure (3.1.1), high accumulation of ^{32}P remained in the main vein of cotyledons after 25 min exposure to the tracer and then ^{32}P was distributed around the cotyledons after 30 min exposure to the tracer. Red and yellow colour indicated high and middle levels of the radioactivity, respectively.

After one hour exposure to the tracer, the translocation of ^{32}P was detected along the hypocotyl. Accumulation of ^{32}P remained high in the area of hypocotyl adjacent to the cotyledons and decreased along the hypocotyl to the roots (Figure 3.1.2). This indicates Pi influx into root. There was no significant difference in the distribution of ^{32}P between 2 mM and 10 mM Pi.

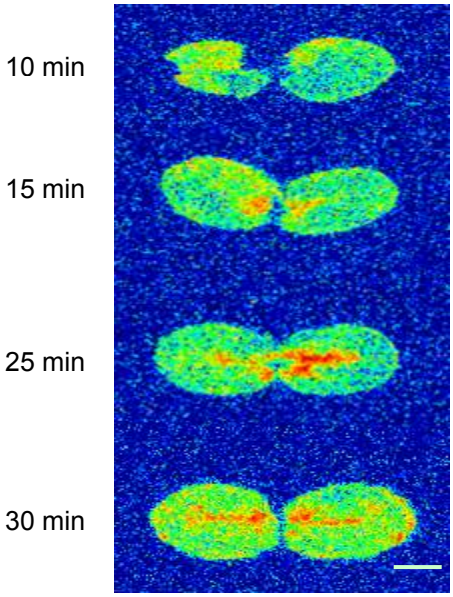


Figure 3.1.1: Autoradiograph of *Ricinus* cotyledons after feeding with ^{32}P. The time on the left side indicates the time the cotyledons were exposed to ^{32}P (Scale bars = 10 mm).

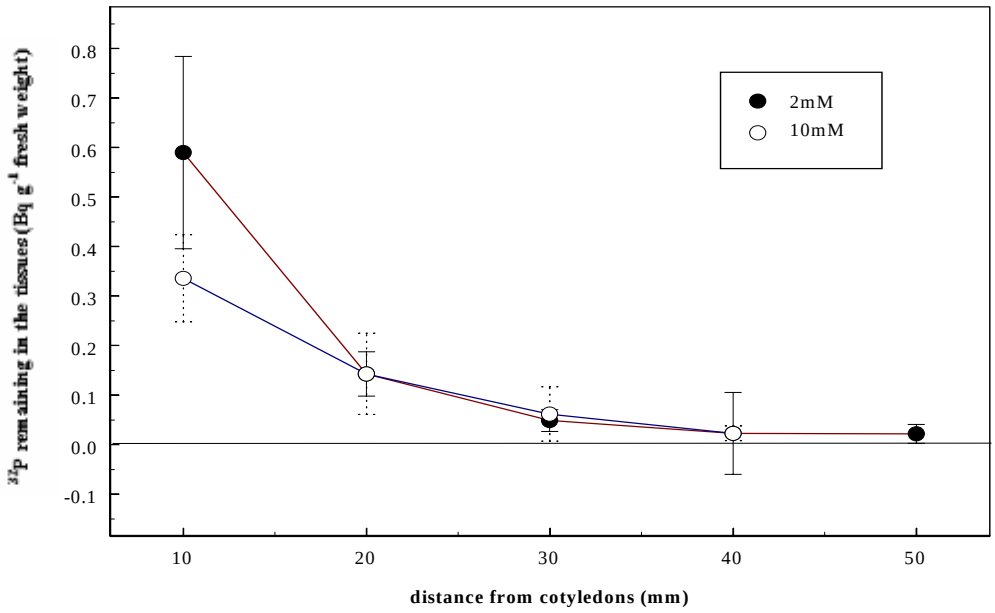


Figure 3.1.2: Distribution of phosphorus tracer (^{32}P) within the hypocotyl after feeding the tracer to the cotyledons for 1 hour. Pi incubation buffers were either 2 mM or 10 mM. Horizontal line is the detection limit of the tracer. Data are means of three replicates ±SD.

3.1.2 Phosphate fluxes after incubation of the roots with phosphate

To examine the possibility of movement of Pi through the xylem via roots, the flux rates were measured by feeding a radioactive tracer to the roots. Roots of seedlings were immersed in an uptake solution containing ^{32}P. The movement of ^{32}P from roots through the hypocotyl was considerable. There were no significant differences in uptake rate of Pi between 2 mM and 10 mM Pi buffers (Table 3.1.1).

Table 3.1.1: Phosphate transport into the hypocotyl after incubation of roots with ^{32}P.

Concentration of P in root buffer	Transport of Pinto the hypocotyl
2 mM	0.042 ± 0.027 µMol h^{-1} $seedling^{-1}$
10 mM	0.052 ± 0.034 µMol h^{-1} $seedling^{-1}$

^{32}P was detected initially in the cotyledons within 10 min after exposure to ^{32}P and had been seen clearly in the minor veins and upper part of hypocotyl after 1 h exposure to ^{32}P. These results indicated that ^{32}P present in the roots was translocated to the hypocotyl and then to the cotyledons. Thereby, the hypocotyl and the cotyledons accumulated large amount of tracer (Figure 3.1.3).

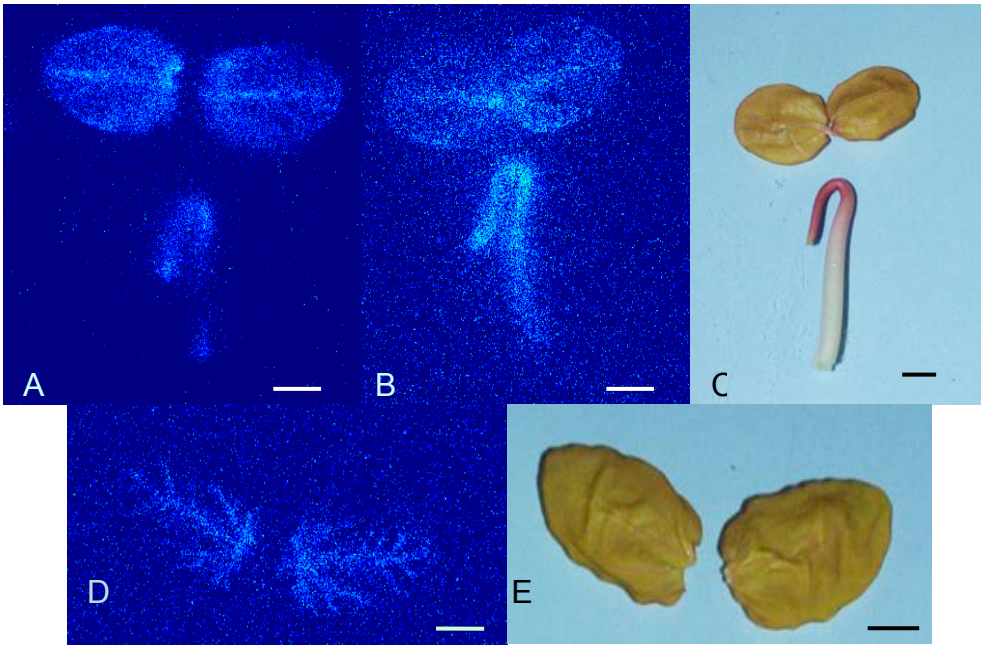


Figure 3.1.3: Autoradiographs of the distribution of phosphate tracer in cotyledons and hypocotyl after seedlings were fed with tracer via the roots (2 mM phosphate buffer). (A) cotyledons and hypocotyl after an incubation time of 10 min. (B) cotyledons and hypocotyl after an incubation time of 60 min, (D) cotyledons after an incubation time of 60 min (C and E) Schematic representation of *Ricinus* seedlings and the cotyledons (Scale bars = 10 mm); roots were removed after incubation time.

The higher concentration of tracer per plant fresh weight was measured in the cotyledons and in the hook of hypocotyl. The specific activity increased markedly within the hypocotyl from base towards the hook. It was highest in the basal parts of the cotyledons (Figure 3.1.4).

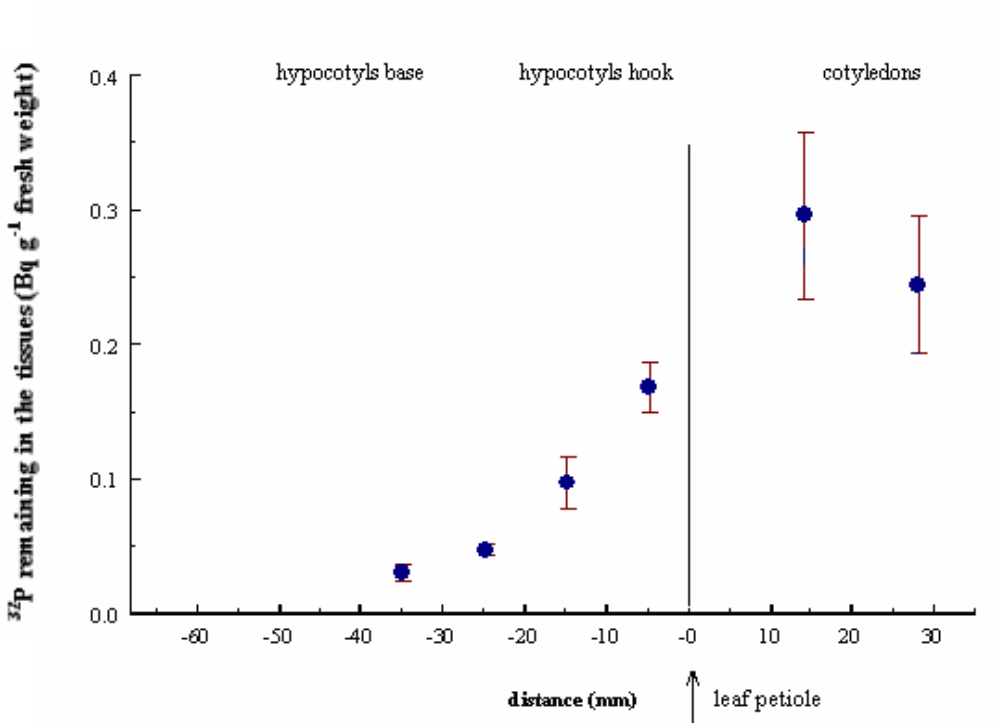


Figure 3.1.4: Distribution of phosphorus tracer after 1 h. Roots were incubated with ^{32}P in a 2 mM phosphate buffer. The arrow indicates the position, where cotyledons join to the hypocotyl. Data are means of three replicates ±SD.

3.2 Effect of phosphate deficiency on germination and growth of *Ricinus* plants

3.2.1 Influence of exogenous Pi on *Ricinus* seedlings during germination

Pi is involved in many reactions in seeds such as formation of various phosphate sugars, nucleotides for metabolism and energy producing processes and synthesis of nucleic acids. Lott *et al*. (1995) suggested that phytic acid constitutes 60 - 80% of the total phosphate content of the seeds. At the beginning of germination, phytic acid is degraded to release free Pi for growth (Carla *et al*., 2001). The experiments were performed to determine if the growth of *Ricinus* seedlings is dependent on exogenous Pi supply in the early phase of germination. The *Ricinus* seedlings were grown in a nutrient medium in the presence of Pi (+Pi) as a control or without Pi (-Pi). The fresh weight and dry weight

of endosperm, cotyledons, hypocotyl and roots were measured from 3- to 7-day-old *Ricinus* seedlings.

The results showed that a reduction in the cotyledons, hypocotyl and root fresh and dry weight were detectable in 6-day-old seedlings, which were grown in Pi deficiency. The highest fresh and dry weight of the hypocotyl and roots were observed in the 7-day-old seedlings grown in Pi sufficiency compared with that in the seedlings grown in Pi deficiency. No significant reduction was detected in the fresh and dry weights of the endosperms under both Pi sufficient or Pi deficient conditions (Figure 3.2.1). The endosperm fresh weight started to decrease during day 6, while dry weight started to decrease at day 4. To determine whether the total Pi concentration of the seedlings grown under Pi sufficient and Pi deficient conditions are changed based on an exogenous Pi supply, samples from separated organs of 7-day-old seedlings were analyzed. The results showed that the total Pi concentration of the seedlings which were grown under Pi sufficient conditions was slightly higher than that in the seedlings grown under Pi deficient conditions. In both conditions, the total Pi concentration of the cotyledons was higher than that in the hypocotyl, roots and endosperm. In addition, the total Pi concentration of the endosperm became very low at this day compared with that of cotyledons, hypocotyl and roots.

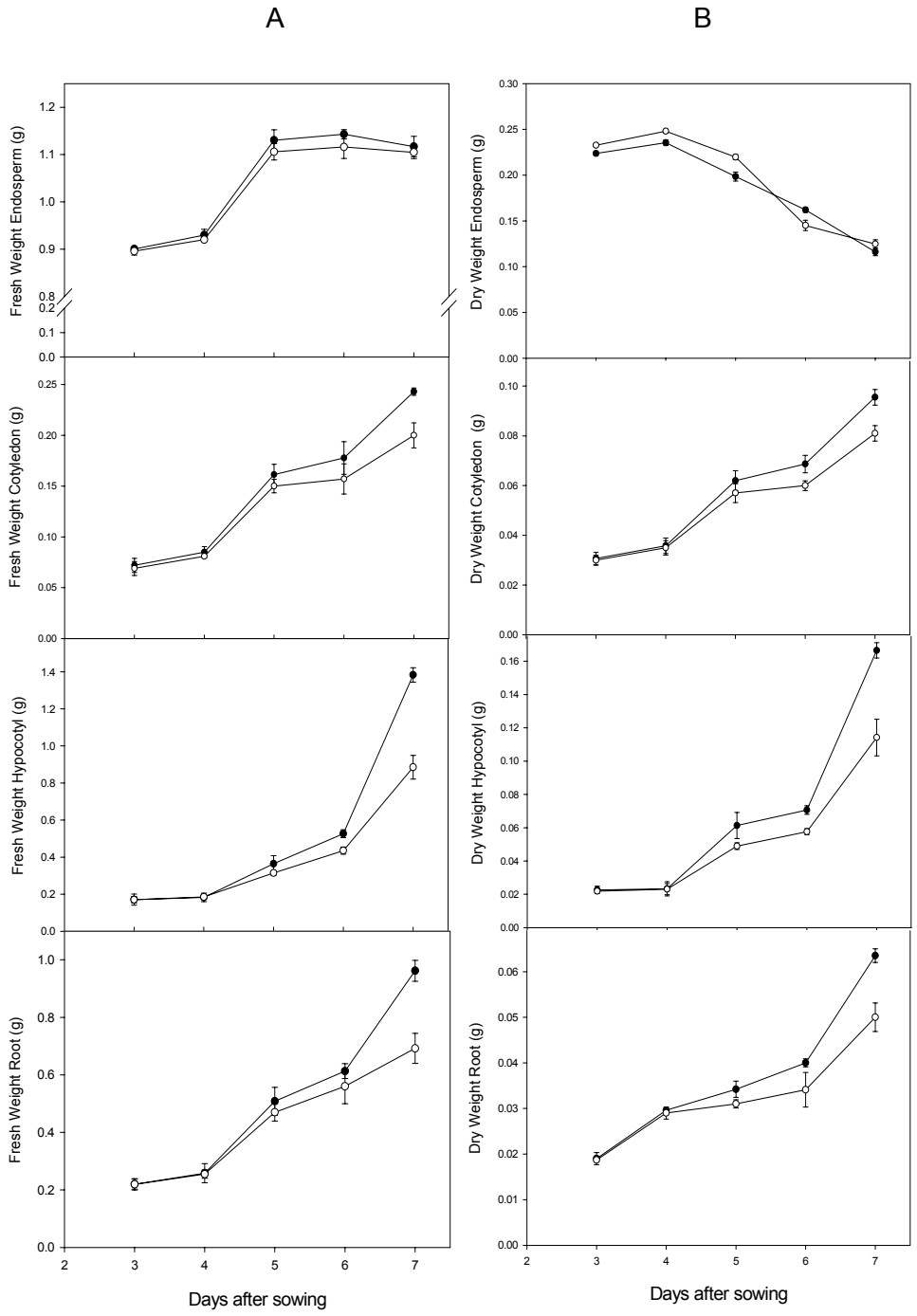


Figure 3.2.1: Changes in fresh and dry weight of the *Ricnius* seedlings grown in the nutrient medium in the presence of Pi (●) and absence of Pi (○). Data are means of five samples ±SD.

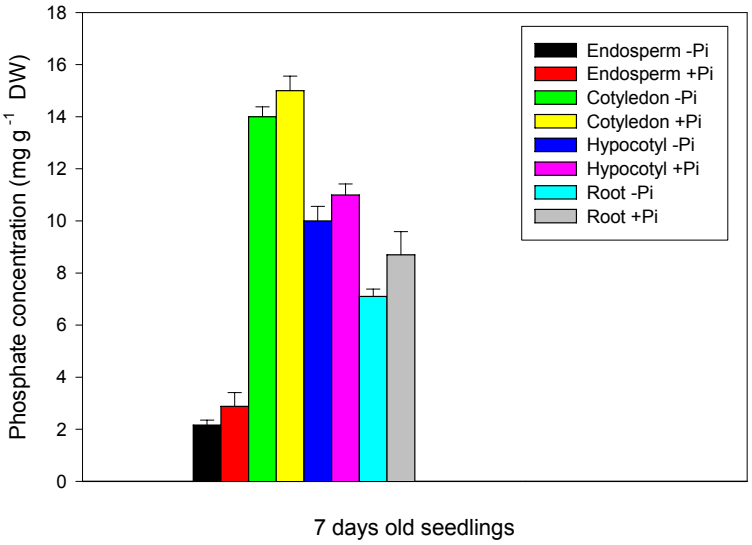


Figure 3.2.2: The total Pi concentration of 7-day-old *Ricinus* seedlings grown in the nutrient medium in the presence of Pi (+Pi) and without Pi (-Pi). Data are means ±SD of five samples.

3.2.2 Effect of Pi deficiency on plant growth and phosphate concentration

To assess the effect of Pi starvation on plants, 7-day-old *Ricinus* seedlings were transferred from nutrient medium in a dark chamber into sterilized quartz sand in the green house and were fertilized daily with Hoagland solution containing either 500 µM Pi or no Pi. After 22 days, Pi starvation led to a severe reduction in growth of *Ricinus* plants; typical deficiency symptoms were recognized in Pi-starved plants such as a darker green colour of the leaf and a smaller leaf surface area than in the control plants. As shown in Figure 3.2.3, the dry weight of leaves and stems of Pi-starved plants were decreased 40% and 45%, respectively, while the dry weight of roots was increased 15% compared with that in the controls. The total Pi concentration was significantly higher in the leaves, stems and roots of the control plants than that of Pi-starved plants (Figure 3.2.3). The total Pi concentration in roots of Pi-starved plants was 3.2 times lower than that obtained from Pi sufficient plants.

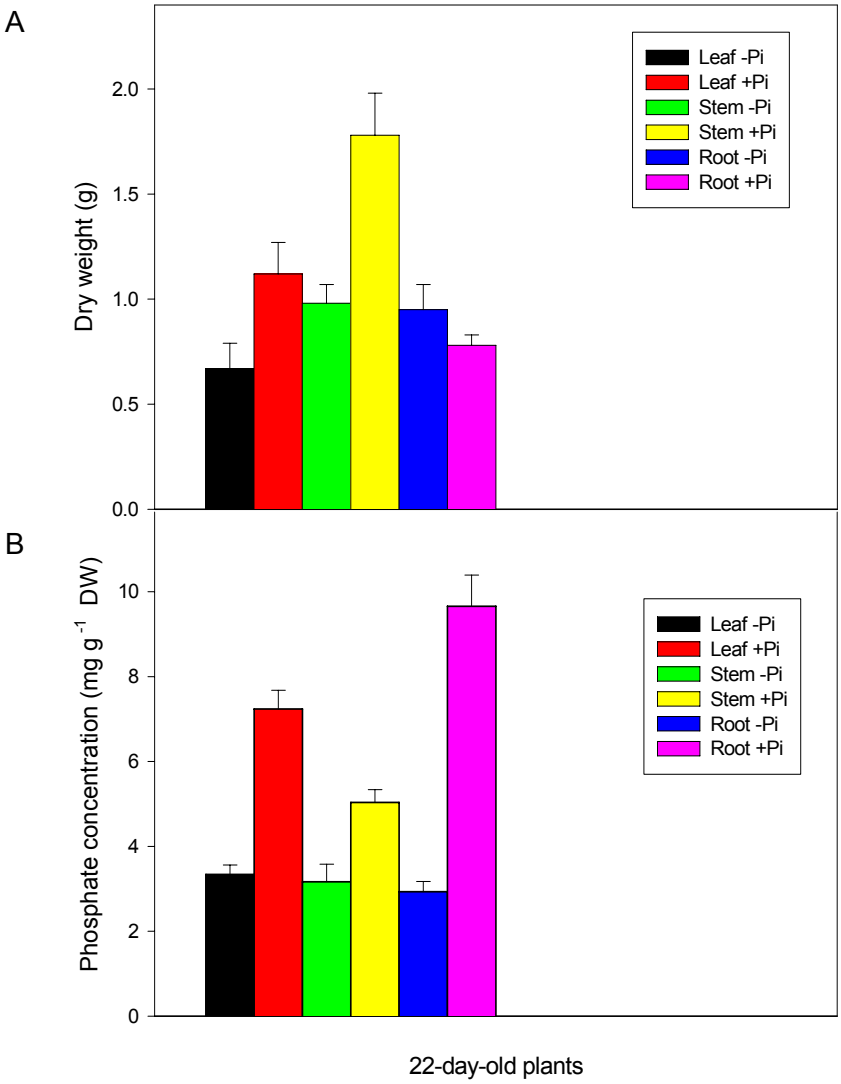


Figure 3.2.3: Influence of low-Pi treatment on the dry weight distribution between plant parts (A) and the total Pi concentrations (B) of 22-day-old *Ricinus* plants grown either in Pi sufficient (+Pi) or Pi deficient (-Pi) conditions. Data are means ±SD of five samples.

3.3 Cloning and functional characterization of RcPT1, a phosphate transporter

Plants have two Pi uptake systems, a low-affinity and a high-affinity transport system. Under Pi starvation condition, the high-affinity system is considered as a major mechanism for Pi uptake by the roots. Numerous genes encoding phosphate transporters have been isolated from different plant species. Expression analysis of these genes showed that the phosphate transporter proteins have distinct functions in Pi uptake, distribution and remobilization (Leggewie *et al*., 1997; Raghothana, 2000; Paszkowski *et al*., 2002).

3.3.1 Cloning of RcPT1, a phosphate transporter

A cDNA fragment of phosphate transporter from *Ricinus* was isolated by using reverse transcriptase polymerase chain reaction (RT-PCR); a pair of degenerate primers consisting of a degenerated forward primer *PTRcF1* (5´-ATG GGW TTY TTY ACW GAT GC-3´) and a degenerated reverse primer *PTRcR1* (5´-CCD AAA TTW GCD AAD AAD AAW-3´) which were designed based on the highly conserved region of the amino acid sequence of several novel phosphate transporter proteins, including AtPT1 (accession number Q96302), LePT1 (accession number O22548), MtPT1 (accession number O22301), StPT1 (accession number Q43650), OsPT11 (accession number AN39052), and PIT1 (accession number O22055), as shown in Figure 3.3.1. A cDNA prepared from 6-day-old cotyledons was used in the PCR reaction. The obtained PCR product from the first PCR with annealing temperature at 42°C was purified by using High Pure PCR product kit (2.1.2) and subsequently used as a template for the second PCR with annealing temperature at 54°C. As optimal condition for successful PCR the following was identified:

1 cycle		94°C	120 sec
30 cycles	{	94°C	60 sec
		54°C	50 sec
		72°C	60 sec
followed by		72°C	10 min

After PCR, a product of approximately 1 kb was cloned in pGEM®-T Easy vector and then sequenced (Figure 3.3.1). The amino acid sequence analysis of obtained fragment showed high similarity to the other phosphate transporter proteins. Based on sequence information of this initial PCR product, gene-specific primers were designed for further cloning of the 3´- and 5´ end of *RcPT1* (Table 2.2B).

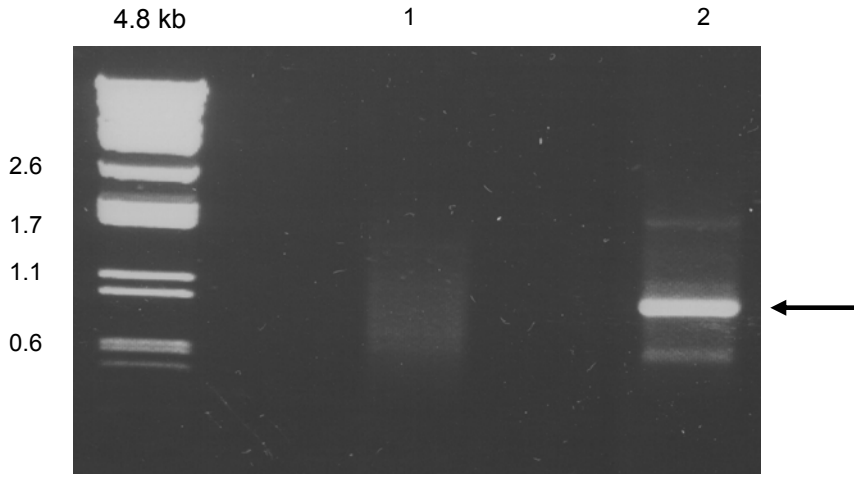
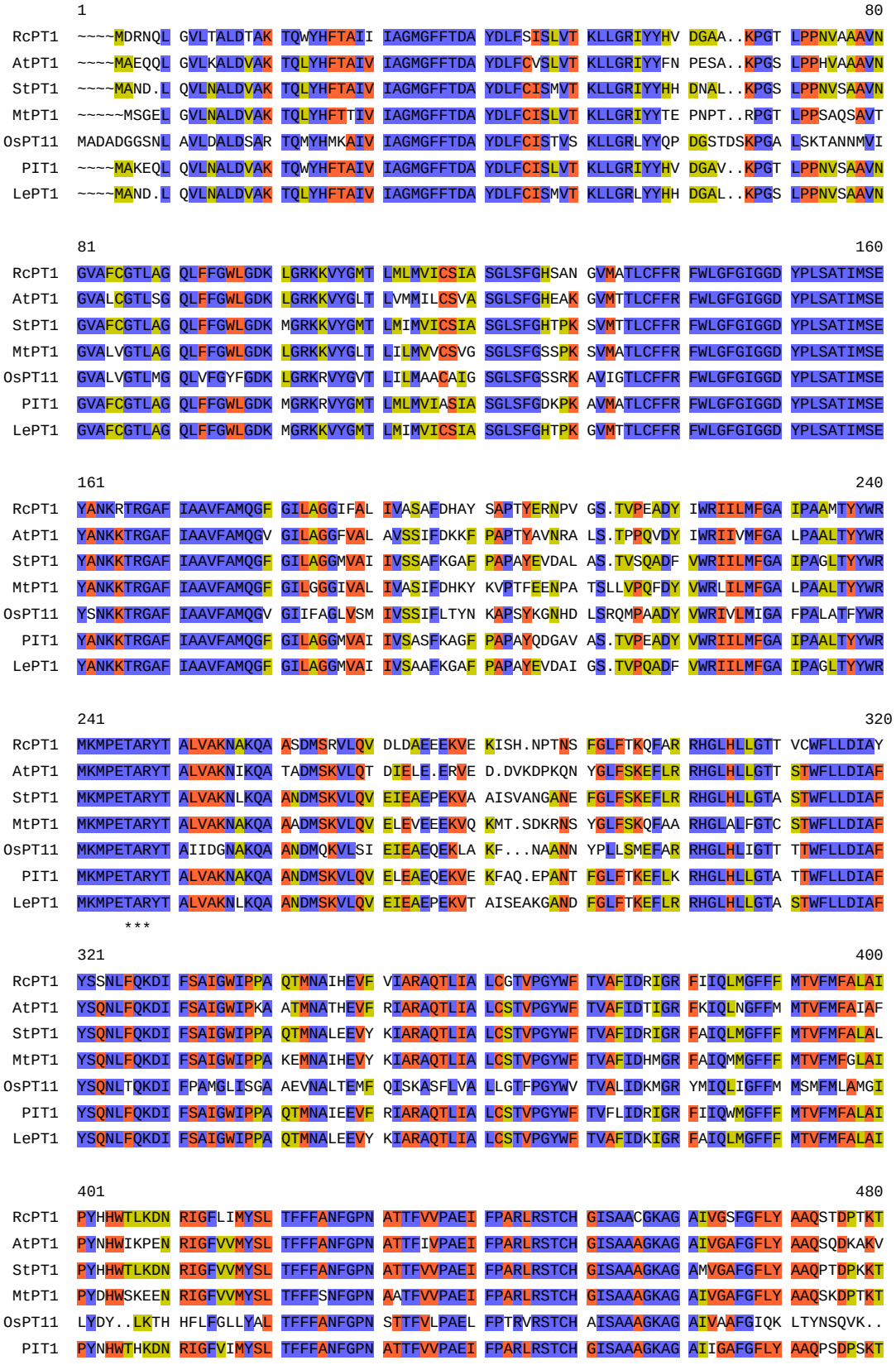


Figure 3.3.1: Amplification of *RcPT1* homologue by degenerate PCR. Lane 1 is the PCR product from the first PCR. Lane 2 is the obtained PCR product of approximately 1 kb. Sizes of relevant bands from the 4.8 kb DNA ladder are shown in kb. The arrow indicates the position of product on the 1% agarose gel.

3.3.2 Deduced peptide sequence of RcPT1 showing a structure of a transmembrane protein

The 5´ and 3´ flanks of *RcPT1* cDNA were cloned by RLM-RACE (2.2.7). 5´ RACE was firstly performed with gene-specific primers *PTRcR1* and 5´ RACE outer primers, then the initial PCR product was further amplified with gene-specific primers *PTRcR2* and 5´ RACE inner primers. The amplified PCR product contained approximately 600 bp. In 3´RACE, a PCR product of 950 bp was amplified with gene-specific primers *PTRcF2* and 3´ RACE inner primers. After obtaining sequence information of the amplification product from 5´ and 3´ RACE, a pair of gene-specific primers *PT-RcF3* and *PT-RcR3* were designed and used to amplify the full-length cDNA.

A


1 80
RcPT1 ~~~~MDRNQL GVLTALDTAK TQWYHFTAII IAGMGFFTDA YDLFSISLVT KLLGRIYYHV DGAA..KPGT LPPNVAAAVN
AtPT1 ~~~~MAEQQL GVLKALDVAK TQLYHFTAIV IAGMGFFTDA YDLFCVSLVT KLLGRIYYFN PESA..KPGS LPPHVAAAVN
StPT1 ~~~~MAND.L QVLNALDVAK TQLYHFTAIV IAGMGFFTDA YDLFCISMVT KLLGRIYYHH DNAL..KPGS LPPNVSAAVN
MtPT1 ~~~~~MSGEL GVLNALDVAK TQLYHFTTIV IAGMGFFTDA YDLFCISLVT KLLGRIYYTE PNPT..RPGT LPPSAQSAVT
OsPT11 MADADGGSNL AVLDALDSAR TQMYHMKAIV IAGMGFFTDA YDLFCISTVS KLLGRLYYQP DGSTDSKPGA LSKTANNMVI
PIT1 ~~~~MAKEQL QVLNALDVAK TQWYHFTAIV IAGMGFFTDA YDLFCISLVT KLLGRIYYHV DGAV..KPGT LPPNVSAAVN
LePT1 ~~~~MAND.L QVLNALDVAK TQLYHFTAIV IAGMGFFTDA YDLFCISMVT KLLGRLYYHH DGAL..KPGS LPPNVSAAVN
81 160
RcPT1 GVAFCGTLAG QLFFGWLGDK LGRKKVYGMT LMLMVICSIA SGLSFGHSAN GVMATLCFFR FWLGFGIGGD YPLSATIMSE
AtPT1 GVALCGTLSG QLFFGWLGDK LGRKKVYGLT LVMMILCSVA SGLSFGHEAK GVMTTLCFFR FWLGFGIGGD YPLSATIMSE
StPT1 GVAFCGTLAG QLFFGWLGDK MGRKKVYGMT LMIMVICSIA SGLSFGHTPK SVMTTLCFFR FWLGFGIGGD YPLSATIMSE
MtPT1 GVALVGTLAG QLFFGWLGDK LGRKKVYGLT LILMVVCSVG SGLSFGSSPK SVMATLCFFR FWLGFGIGGD YPLSATIMSE
OsPT11 GVALVGTLMG QLVFGYFGDK LGRKRVYGVT LILMAACAIG SGLSFGSSRK AVIGTLCFFR FWLGFGIGGD YPLSATIMSE
PIT1 GVAFCGTLAG QLFFGWLGDK MGRKRVYGMT LMLMVIASIA SGLSFGDKPK AVMATLCFFR FWLGFGIGGD YPLSATIMSE
LePT1 GVAFCGTLAG QLFFGWLGDK MGRKKVYGMT LMIMVICSIA SGLSFGHTPK GVMTTLCFFR FWLGFGIGGD YPLSATIMSE
161 240
RcPT1 YANKRTRGAF IAAVFAMQGF GILAGGIFAL IVASAFDHAY SAPTYERNPV GS.TVPEADY IWRIILMFGA IPAAMTYYWR
AtPT1 YANKKTRGAF IAAVFAMQGV GILAGGFVAL AVSSIFDKKF PAPTYAVNRA LS.TPPQVDY IWRIIVMFGA LPAALTYYWR
StPT1 YANKKTRGAF IAAVFAMQGF GILAGGMVAI IVSSAFKGAF PAPAYEVDAL AS.TVSQADF VWRIILMFGA IPAGLTYYWR
MtPT1 YANKKTRGAF IAAVFAMQGF GILGGGIVAL IVASIFDHKY KVPTFEENPA TSLLVPQFDY VWRLILMFGA LPAALTYYWR
OsPT11 YSNKKTRGAF IAAVFAMQGV GIIFAGLVSM IVSSIFLTYN KAPSYKGNHD LSRQMPAADY VWRIVLMIGA FPALATFYWR
PIT1 YANKKTRGAF IAAVFAMQGF GILAGGMVAI IVSASFKAGF PAPAYQDGAV AS.TVPEADY VWRIILMFGA IPAALTYYWR
LePT1 YANKKTRGAF IAAVFAMQGF GILAGGMVAI IVSAAFKGAF PAPAYEVDAI GS.TVPQADF VWRIILMFGA IPAGLTYYWR
241 320
RcPT1 MKMPETARYT ALVAKNAKQA ASDMSRVLQV DLDAEEEKVE KISH.NPTNS FGLFTKQFAR RHGLHLLGTT VCWFLLDIAY
AtPT1 MKMPETARYT ALVAKNIKQA TADMSKVLQT DIELE.ERVE D.DVKDPKQN YGLFSKEFLR RHGLHLLGTT STWFLLDIAF
StPT1 MKMPETARYT ALVAKNLKQA ANDMSKVLQV EIEAEPEKVA AISVANGANE FGLFSKEFLR RHGLHLLGTA STWFLLDIAF
MtPT1 MKMPETARYT ALVAKNAKQA AADMSKVLQV ELEVEEEKVQ KMT.SDKRNS YGLFSKQFAA RHGLALFGTC STWFLLDIAF
OsPT11 MKMPETARYT AIIDGNAKQA ANDMQKVLSI EIEAEQEKLA KF...NAANN YPLLSMEFAR RHGLHLIGTT TTWFLLDIAF
PIT1 MKMPETARYT ALVAKNAKQA ANDMSKVLQV ELEAEQEKVE KFAQ.EPANT FGLFTKEFLK RHGLHLLGTA TTWFLLDIAF
LePT1 MKMPETARYT ALVAKNLKQA ANDMSKVLQV EIEAEPEKVT AISEAKGAND FGLFTKEFLR RHGLHLLGTA STWFLLDIAF
***
321 400
RcPT1 YSSNLFQKDI FSAIGWIPPA QTMNAIHEVF VIARAQTLIA LCGTVPGYWF TVAFIDRIGR FIIQLMGFFF MTVFMFALAI
AtPT1 YSQNLFQKDI FSAIGWIPKA ATMNATHEVF RIARAQTLIA LCSTVPGYWF TVAFIDTIGR FKIQLNGFFM MTVFMFAIAF
StPT1 YSQNLFQKDI FSAIGWIPPA QTMNALEEVY KIARAQTLIA LCSTVPGYWF TVAFIDRIGR FAIQLMGFFF MTVFMFALAL
MtPT1 YSQNLFQKDI FSAIGWIPPA KEMNAIHEVY KIARAQTLIA LCSTVPGYWF TVAFIDHMGR FAIQMMGFFF MTVFMFGLAI
OsPT11 YSQNLTQKDI FPAMGLISGA AEVNALTEMF QISKASFLVA LLGTFPGYWV TVALIDKMGR YMIQLIGFFM MSMFMLAMGI
PIT1 YSQNLFQKDI FSAIGWIPPA QTMNAIEEVF RIARAQTLIA LCSTVPGYWF TVFLIDRIGR FIIQWMGFFF MTVFMFALAI
LePT1 YSQNLFQKDI FSAIGWIPPA QTMNALEEVY KIARAQTLIA LCSTVPGYWF TVAFIDKIGR FAIQLMGFFF MTVFMFALAI
401 480
RcPT1 PYHHWTLKDN RIGFLIMYSL TFFFANFGPN ATTFVVPAEI FPARLRSTCH GISAACGKAG AIVGSFGFLY AAQSTDPTKT
AtPT1 PYNHWIKPEN RIGFVVMYSL TFFFANFGPN ATTFIVPAEI FPARLRSTCH GISAAAGKAG AIVGAFGFLY AAQSQDKAKV
StPT1 PYHHWTLKDN RIGFVVMYSL TFFFANFGPN ATTFVVPAEI FPARLRSTCH GISAAAGKAG AMVGAFGFLY AAQPTDPKKT
MtPT1 PYDHWSKEEN RIGFVVMYSL TFFFSNFGPN AATFVVPAEI FPARLRSTCH GISAAAGKAG AIVGAFGFLY AAQSKDPTKT
OsPT11 LYDY..LKTH HFLFGLLYAL TFFFANFGPN STTFVLPAEL FPTRVRSTCH AISAAAGKAG AIVAAFGIQK LTYNSQVK..
PIT1 PYNHWTHKDN RIGFVIMYSL TFFFANFGPN ATTFVVPAEI FPARLRSTCH GISAAAGKAG AIIGAFGFLY AAQPSDPSKT

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LePT1   PYHHWTLKDH RIGFVVMYSF TFFFANFGPN ATTFVVPAEI FPARLRSTCH GISAAAGKAG AMVGAFGFLY AAQPTDPTKT
                                      + +++
        481                                                                                  560
RcPT1   DAGYPTGIGM KNSLIALGVI NFFGILFTLL VPESKGKSLE ELTGE..NED EPQ..... EEAISSRTVP V*~~~~~~~~~
AtPT1   DAGYPPGIGV KNSLIMLGVL NFIGMLFTFL VPEPKGKSLE ELSGE..AEV SHDEK*~~~~~ ~~~~~~~~~~ ~~~~~~~~~~
StPT1   DAGYPAGIGV RNSLIVLGCV NFLGMLFTFL VPESKGKSLE EMSRE..NEG EEETVAEMRA TSG...RTVL FKF*~~~~~~~
MtPT1   DKGYPTGIGI KNSLIMLGVI NFVGMLCTLL VPESKGKSLE ELSGE..NEG EGAEATEQEG PRFENVA*~~~ ~~~~~~~~~~
OsPT11  ........SI KKALIILSIT NMLGFFFTFL VPETMGRSLE EISGEDGNTG AGGGGAPAAA NAGVGVSASD VSRDEKFPAS*
 PIT1   DKGYPPGIGV KNALIVLGCV NFLGMVFTFL VPEAKGKSLE EVSKE..NEE EVENGTELRQ QSGHDTRTVP VL*~~~~~~~~
LePT1   DAGYPPGIGV RNSLIVLGCV NFLGMLFTFL VPESNGKSLE DLSRE..NEG EEETVAEIRA TSG...RTVP V*~~~~~~~~~
                                        *** *
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B

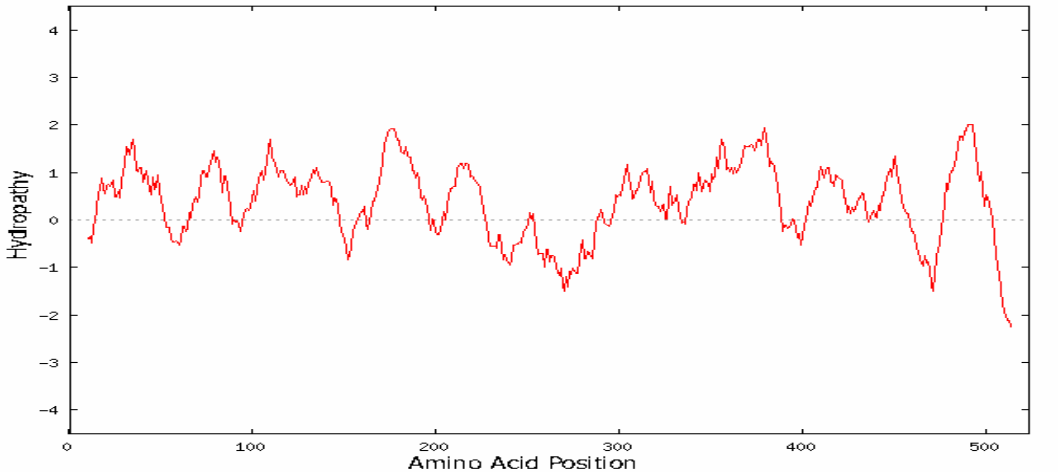


Figure 3.3.2: (A) Alignment of the deduced amino acid sequence of RcPT1 with those of *Arabidopsis thaliana* (AtPT1, accession number Q96302), *Lycopersicon esculentum* (LePT1, accession number O22548), *Medicago truncatula* (MtPT1, accession number O22301), *Solanum tuberosum* (StPT1, accession number Q43650), *Oryza sativa* (OsPT11, accession number AN39052), *Catharanthus roseus* (PIT1, accession number O22055). Identical amino acids are shaded in blue, similar amino acids are shaded in red, not similar amino acids are shaded in yellow. Asterisks within the sequence indicate the stop codon in the nucleotide sequence. Asterisks (***) under the sequence indicate a putative phosphorylation site by protein kinase C, and (****) indicate a putative phosphorylation site by casein kinase II. Pluses under the sequence (++++) indicate a consensus site for N-glycosylation. (B) Hydropathy profile of RcPT1 protein. Hydropathy values for a window size of 7 amino acids were calculated by the method of Kyte and Dootline (Kyte *et al.*, 1982)

The full-length *RcPT1* cDNA is 1867 bp long and contains an open reading frame encoding a 534 amino acid polypeptide (molecular mass of 59 kD). The open reading frame of *RcPT1* is flanked with 102 bp of untranslated sequence at the 5´end and 158 bp of untranslated sequence including poly (A^+) tail at the 3´end. A homology search of the sequence data bases revealed that RcPT1 protein has high similarity with the other phosphate transporters from different plants. *RcPT1* shares 78%, 76%, 80%, 81% , 92.8% amino acid identity with phosphate transporters identified in AtPT1 (accession number Q96302), LePT1 (accession number O22548), MtPT1 (accession number O22301), StPT1 (accession number Q43650), AtPT2 (accession number Q96303) respectively.

Hydropathy plots of the deduced polypeptides showed that both integral membrane proteins consisted of 12 membrane spanning domains. Six N-terminal and six C-terminal domains are separated by a central hydrophilic domain. This common feature is shared by other membrane associated co-transporters which are responsible for transport of substrates as diverse as sugars, ions, antibiotics, and amino acids (Griffith *et al*, 1992; Henderson, 1993; Marger and Saier, 1993). The position and spacing of these membrane-spanning regions are very similar to those of other Pi transporters.

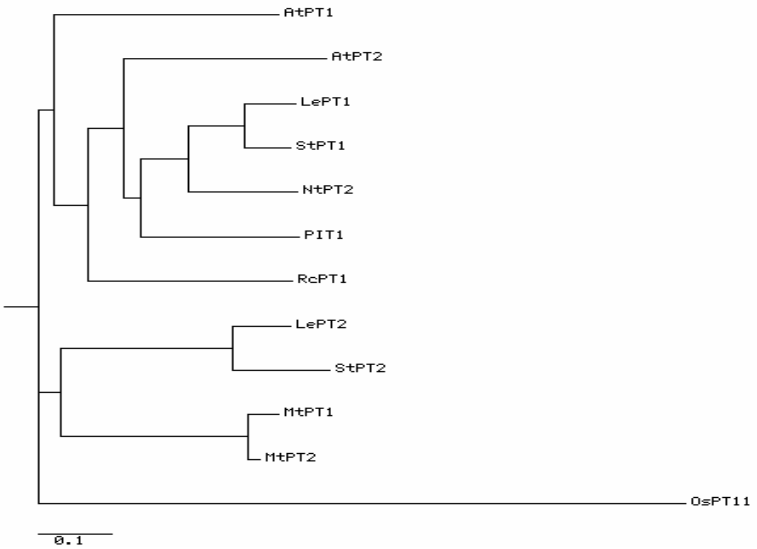
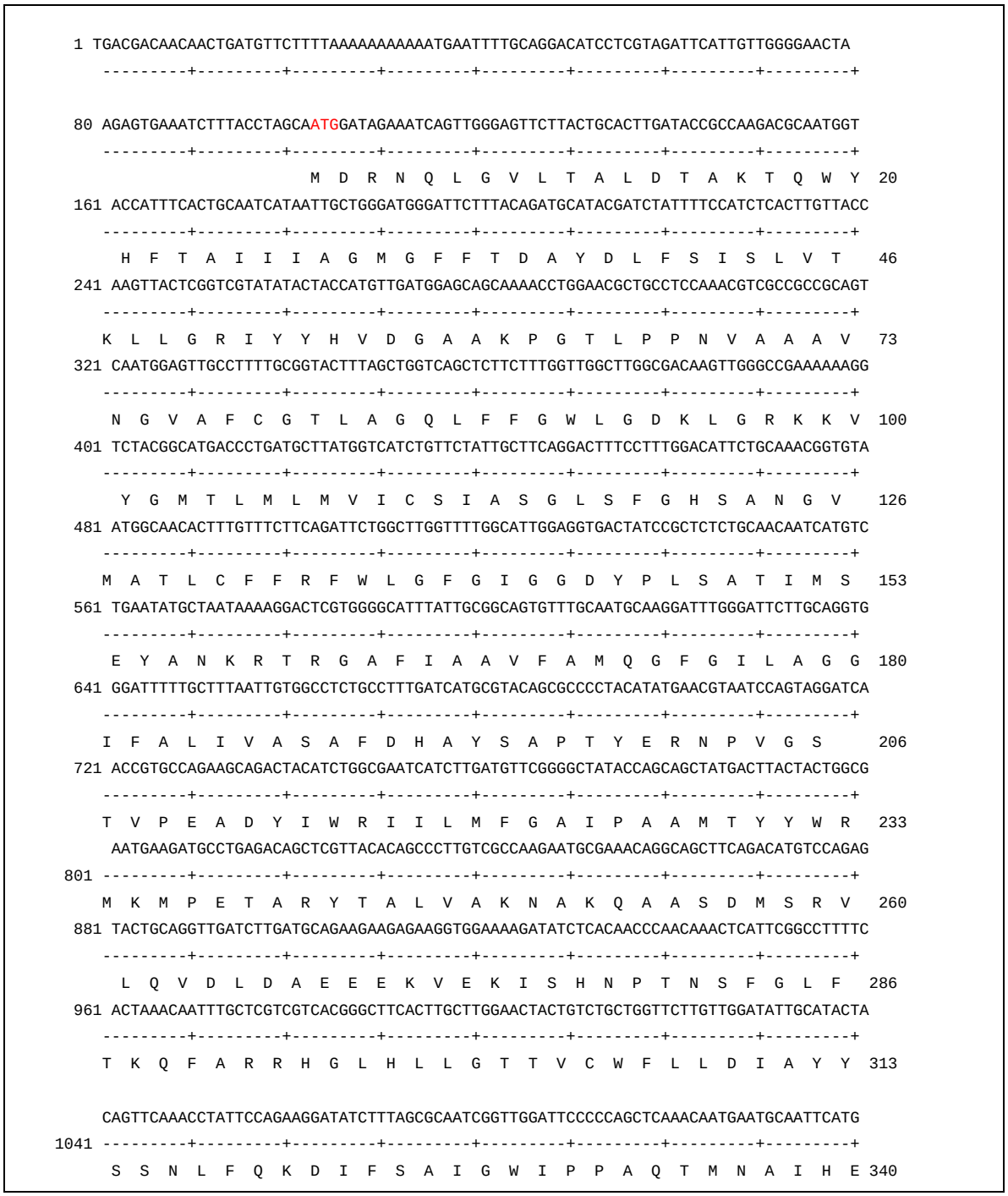


Figure 3.3.3: Phylogenetic relationship of RcPT1 and other phosphate transporters.
Phylogenetic analysis of RcPT1 and related high-affinity Pi transporters from *Arabidopsis thaliana* (AtPT1, accession number Q96302) and AtPT2 (accession number Q96303), *Lycopersicon*

esculentum LePT1 (accession number O22548) and LePT2 (accession number O22549), *Medicago truncatula* MtPT1 (accession number O22301) and MtPT2 (accession number O22302), *Solanum tuberosum* StPT1 (accession number Q43650) and StPT2 (accession number Q41479), *Catharanthus roseus* PIT1(accession number O22055), *Nicotiana tabacum* NtPT2 (accession number Q9LLS5), *Oryza sativa* OstPT11. Sequence divergence are related to branch lengths and can be estimated relative to the 0.1 bar shown.



```
1121 AAGTCTTTGTTATTGCAAGAGCACAGACACTTATCGCCTTATGCGGTACAGTTCCTGGATACTGGTTTACAGTGGCTTTT
     ---------+---------+---------+---------+---------+---------+---------+---------+
       V  F  V  I  A  R  A  Q  T  L  I  A  L  C  G  T  V  P  G  Y  W  F  T  V  A  F   366
 1201 ATTGATAGAATAGGAAGGTTTATCATCCAATTGATGGGATTCTTCTTCATGACTGTGTTTATGTTTGCGCTGGCAATACC
     ---------+---------+---------+---------+---------+---------+---------+---------+
     I  D  R  I  G  R  F  I  I  Q  L  M  G  F  F  F  M  T  V  F  M  F  A  L  A  I  P  393
 1281 TTATCATCACTGGACACTGAAGGATAACAGAATTGGGTTCTTGATCATGTACTCACTGACATTCTTCTTCGCGAATTTTG
     ---------+---------+---------+---------+---------+---------+---------+---------+
      Y  H  H  W  T  L  K  D  N  R  I  G  F  L  I  M  Y  S  L  T  F  F  F  A  N  F  G 420
 1361 GACCCAATGCCACAACATTTGTAGTGCCAGCTGAGATTTTCCCAGCAAGATTAAGGTCAACTTGCCATGGAATATCAGCA
     ---------+---------+---------+---------+---------+---------+---------+---------+
       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   446
 1441 GCTTGTGGAAAGGCAGGTGCTATTGTTGGTTCATTTGGGTTCTTGTATGCTGCACAGAGTACTGACCCTACAAAGACTGA
     ---------+---------+---------+---------+---------+---------+---------+---------+
     A  C  G  K  A  G  A  I  V  G  S  F  G  F  L  Y  A  A  Q  S  T  D  P  T  K  T  D  473
 1521 TGCTGGTTATCCAACTGGGATCGGAATGAAAAATTCACTTATTGCGCTTGGTGTGATCAATTTCTTTGGAATTTTGTTCA
     ---------+---------+---------+---------+---------+---------+---------+---------+
       A  G  Y  P  T  G  I  G  M  K  N  S  L  I  A  L  G  V  I  N  F  F  G  I  L  F  T 500
 1601 CTCTGTTGGTGCCCGAGTCAAAGGGGAAATCTCTGGAGGAGCTAACAGGAGAAAATGAGGATGAACCACAAGAAGAAGCT
     ---------+---------+---------+---------+---------+---------+---------+---------+
       L  L  V  P  E  S  K  G  K  S  L  E  E  L  T  G  E  N  E  D  E  P  Q  E  E  A   526
 1681 ATTTCAAGTAGGACAGTTCCTGTATGATTTGGCTACTATAAAGAAGGTATTATCTTGTAATGGAAAATTAATGTAACTTC
     ---------+---------+---------+---------+---------+---------+---------+---------+
      I  S  S  R  T  V  P  V  *                                                       534
 1761 TCAGGTTTTAGTTTATCATGCCTCTCTGTGCTGAATAAGGTATCGACATAATGTAAGAATATATATATAGTGCAATCTCA
     ---------+---------+---------+---------+---------+---------+---------+---------+
 1841 GATCCATTTAGAAAAAAAAAAAGAAAA 1867
     ---------+---------+-------
```

Figure 3.3.4: The nucleotide and amino acid sequences of the full-length *RcPT1* cDNA. Red shade indicates start and stop codon.

3.3.3 Genomic organization of RcPT1 gene

Southern blot was performed to estimate the number of sequences present in the *Ricinus* genome that are homologous to *RcPT1* cDNA. Total genomic DNA of *Ricinus* seedling was digested with restriction enzymes *EcoRV* and *NcoI* (both enzymes contain restriction sites within *RcPT1* cDNA sequence) and *BamHI* and *XbaI* (they have no restriction sites within *RcPT1* cDNA sequence). The full length of *RcPT1* cDNA was generated by PCR with the two gene-specific primers *PT-RcF3* and *PT-RcR3* and used as a template for synthesis of digoxigenin (Dig)-labelled RNA probe (2.2.8).

The blot was washed at high stringency and showed the presence of two bands when the DNA was cut with *EcoRI* and *NcoI*, whereas *BamHI* and *XbaI* generated only one single band. The results suggested that *RcPT1* is present as a single or low-copy gene in the haploid *Ricinus* genome.

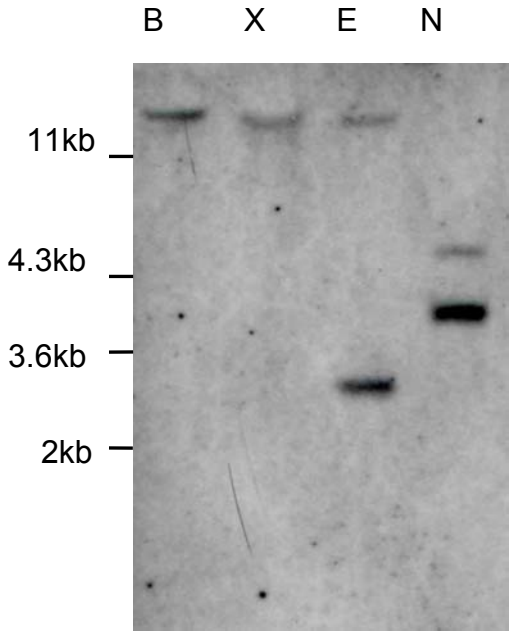


Figure 3.3.5: Southern-blot analysis of *RcPT1*. *Ricinus* genomic DNA digested with *BamHI* (B), *XbaI* (X), *EcoRV* (E), *NcoI* (N). The position of DNA marker fragments and their lengths in kb are indicated at the left side.

3.3.4 Yeast functional complementation and phosphate uptake properties

In order to examine the functional phosphate transport of *RcPT1*, the full length of cDNA *RcPT1* was constructed into p118A1NE vector under the control of the alcohol dehydrogenase promoter and introduced into a yeast phosphate transport mutant strain (MB192). The yeast strain MB192 contains a mutation in the PHO84 gene, thus lacks the high-affinity phosphate transport functions (Bun-ya *et al*., 1991). This yeast mutant showed reduced rates of growth in low-phosphate medium and expresses a Pi-repressible acid phosphatase (rAPase; EC 3.1.3.2). The full length of *PHO84* gene was

also cloned into p118A1NE vector and used as a positive control. Transformants of MB192 were named Y-RcPT1 (p118A1NE vector with *RcPT1*), Y-PHO84 (p118A1NE vector with *PHO84*) and Y-MB (transformant containing only p118A1NE vector). Although, the growth of Y-PHO84 was slightly higher than the growth of Y-RcPT1 after 35 hours, growth of both was faster than of Y-MB (negative control). This indicates that the expression of *RcPT1* increased uptake of phosphate into the cells (Figure 3.3.6).

Yeast mutant cells continued to produce an acid phosphatase (APase) in high-Pi medium, whereas this activity was repressed in wild type in the same condition. In order to examine the function of RcPT1 in yeast mutant cells, Y-RcPT1, Y-PHO84 and Y-MB transformants were grown on phosphatase staining medium. Transformants Y-MB exhibited acid phosphatase activity and their colour turned red after staining, whereas transformants Y-RcPT1 and Y-PHO84 remained white (Figure 3.3.6) indicating that no acid phosphatase activity was present. Moreover, function of the RcPT1 protein was determined by ^{32}P-uptake experiments. The MB192 transformants expressing *RcPT1* displayed a 13.2-fold higher rate of phosphate uptake than the control with empty vector. The transport of Pi into yeast cells mediated by RcPT1 followed Michaelis-Menten kinetics, exhibiting an apparent K_m for Pi of 112 µM.

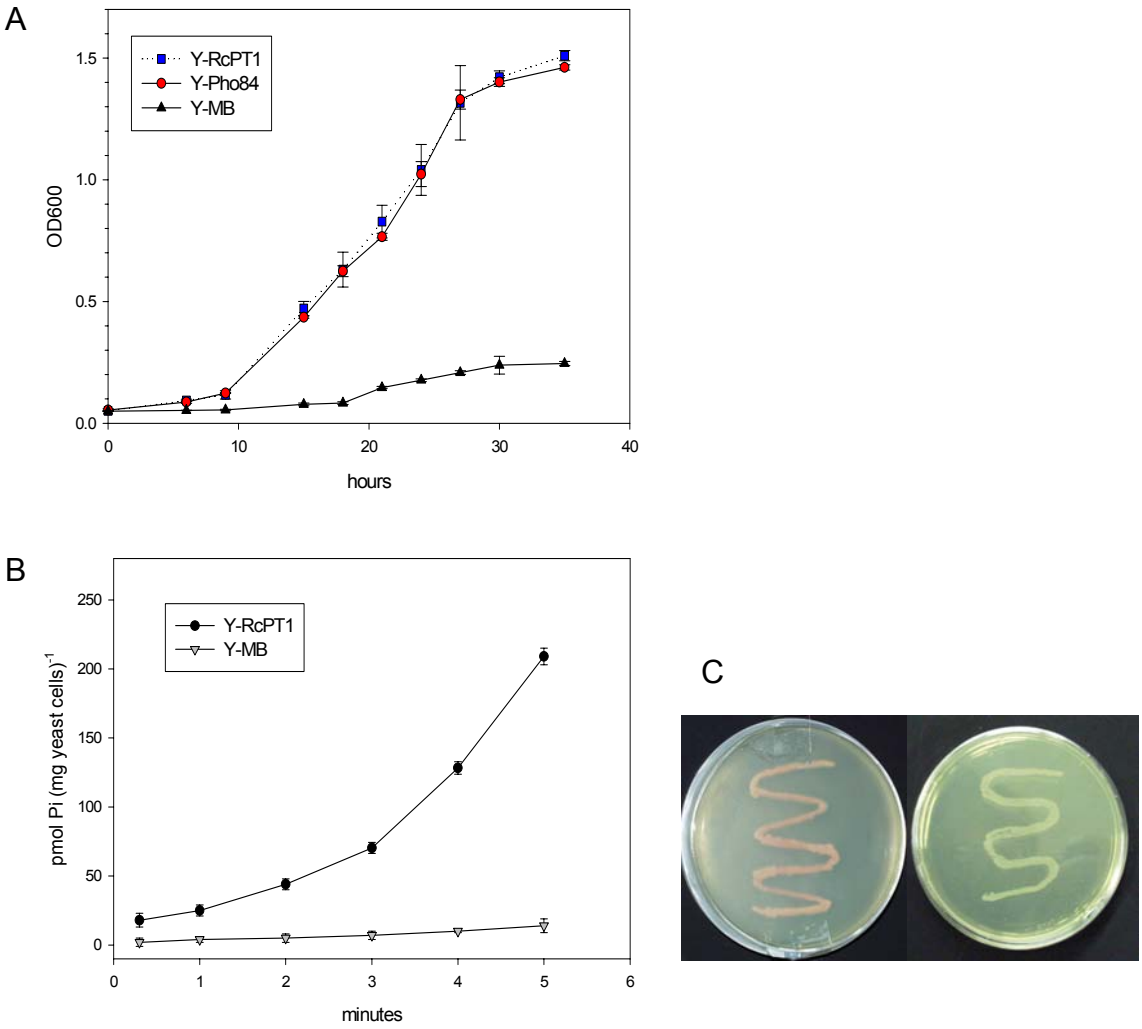


Figure 3.3.6: Functional analysis in yeast. (A) Complementation of yeast high-affinity phosphate transporter mutant MB192. (B) Uptake of Pi by yeast cells. Transformants containing vector p118A1NE with *RcPT1* (Y-RcPT1), transformants containing vector p118A1NE with *Pho84* (Y-Pho84), transformants containing empty vector p118A1NE (Y-MB). (C) Acid phosphatase activity test of yeast phosphate transporter mutant: yeast expression vector p118A1NE without insert Y-MB (left), yeast expression vector p118A1NE containing *RcPT1* (right). Pi-starved cells were incubated at 30°C for five minutes in presence of ^{32}P.

3.3.5 Expression of phosphate transporter RcPT1 during germination of seedlings

Since the beginning of germination, Pi is transported from the endosperm to the growing seedling. To examine the spatial and temporal expression of phosphate transporter *RcPT1* gene in relation to growth of seedlings during germination, total RNA was isolated from different tissues from 3 to 7-day-old seedlings grown in the presence of 500µM (+Pi) or no Pi (-Pi) and was separated on gel. The expression of *RcPT1* was detected by Northern-blot with Dig-labelled RNA 3´-untranslated region (UTR) of *RcPT1*.

In order to synthesize a 3´-UTR of *RcPT1* probe, a DNA fragment approximately 140 bp was generated by PCR with specific primers *PTRcF4* and *PTRcR3*, which are complementary to the sequences downstream of the stop codon and 3´ untranslated region of *RcPT1* cDNA, respectively (Table 2.2.2 B). This PCR fragment was then cloned into pGEM®-T Easy. The RNA probes were synthesized as described in 2.2.9. The anti-sense probe was obtained by using T7-RNA polymerase, while sense probe was obtained by using SP6-RNA polymerase. This sense probe was used for further experiments and *in situ* hybridization.

Hybridization with 3´-UTR of *RcPT1* probe showed that 3 days after sowing, accumulation of *RcPT1* was first detected in the hypocotyl and the roots, even though it was at low level. The signal increased until 6 days of germination in both tissues. In the endosperm, *RcPT1* transcripts were detected after 4 days and increased until 6 days and then decreased to a low level at 7 days. *RcPT1* was only expressed in cotyledons at 6 days and transcript levels increased at 7 days. At 7 days, *RcPT1* transcript accumulation was higher in roots than in cotyledons and hypocotyls, while *RcPT1* transcripts were not detectable in endosperm (Figure 3.3.7).

Although, during germination the transcript levels of *RcPT1* were different between endosperm, cotyledons, hypocotyl and roots, similar transcript levels were obtained in each tissue whether the seedlings were grown in -Pi and +Pi conditions.

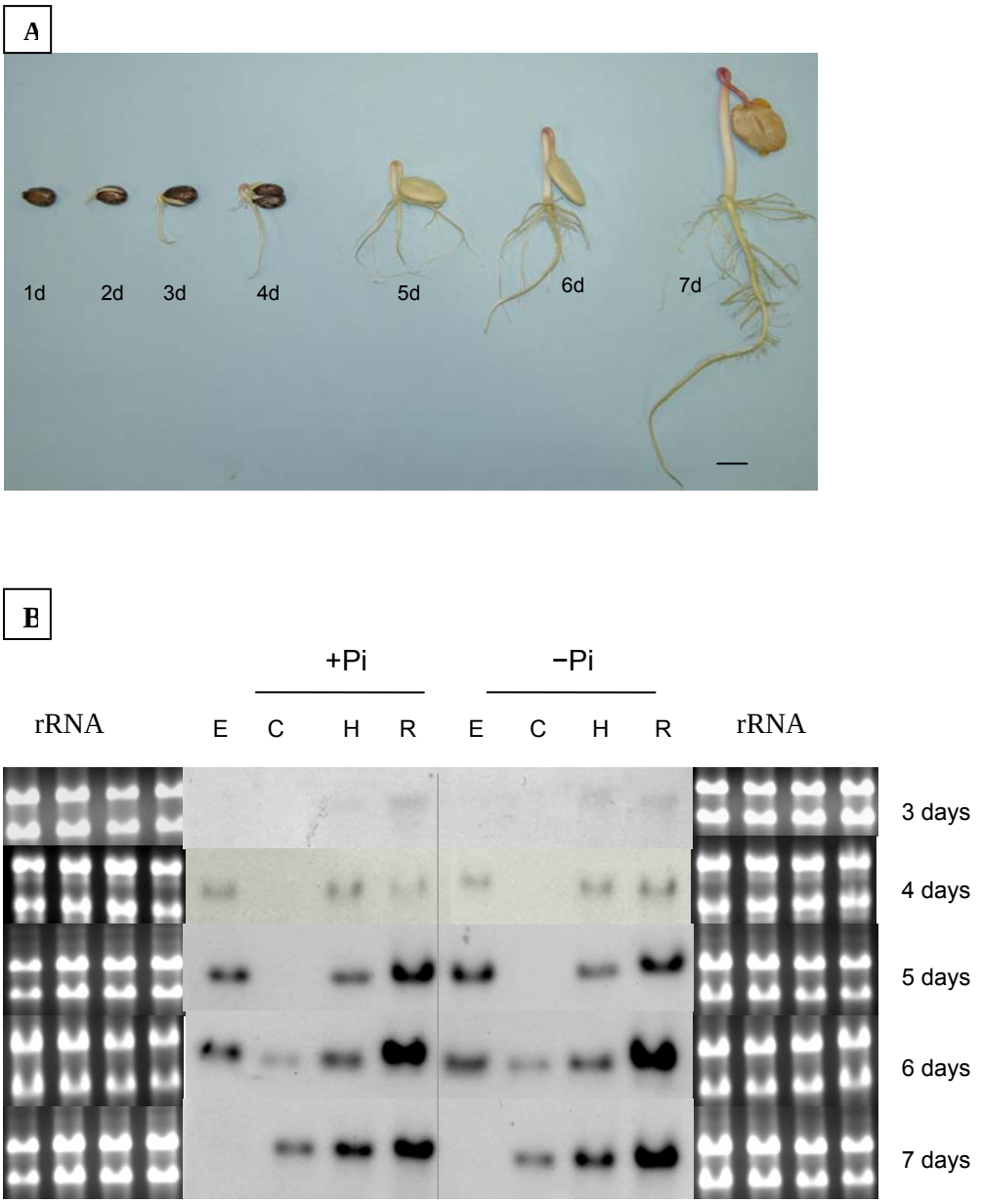


Figure 3.3.7: Northern blot analysis of phosphate transporter *RcPT1* gene in different organs at various stages of germination. (A) Schematic representation of *Ricinus* seedlings from 1 to 7 days after sowing (Scale bar = 1 cm). (B) Total RNA from endosperm (E); cotyledons (C); hypocotyl (H); roots (R) of *Ricinus* seedlings grown in either 500 µM Pi (+Pi) or no Pi (−Pi). RNA extracts were hybridized to anti-sense probe corresponding to 3´ untranslated region (UTR) of *RcPT1* cDNA. 10 µg of total RNA was subjected to Northern blot analysis. Gel pictures in the left and the right side of blot show uniform loading of RNA.

3.3.6 Western blot analysis of the RcPT1 protein in seedling

In order to determine whether the RcPT1 protein levels correspond to the transcript levels, Western blot analysis was performed. The total proteins were isolated from endosperm, cotyledons, hypocotyl and roots of 7-day-old *Ricinus* seedlings grown in Pi deficient condition. The result indicated that the antibody reacted specifically with a 59-kD protein present in roots, hypocotyl and cotyledons of seedling, while no signal was detected in endosperm (3.3.8).

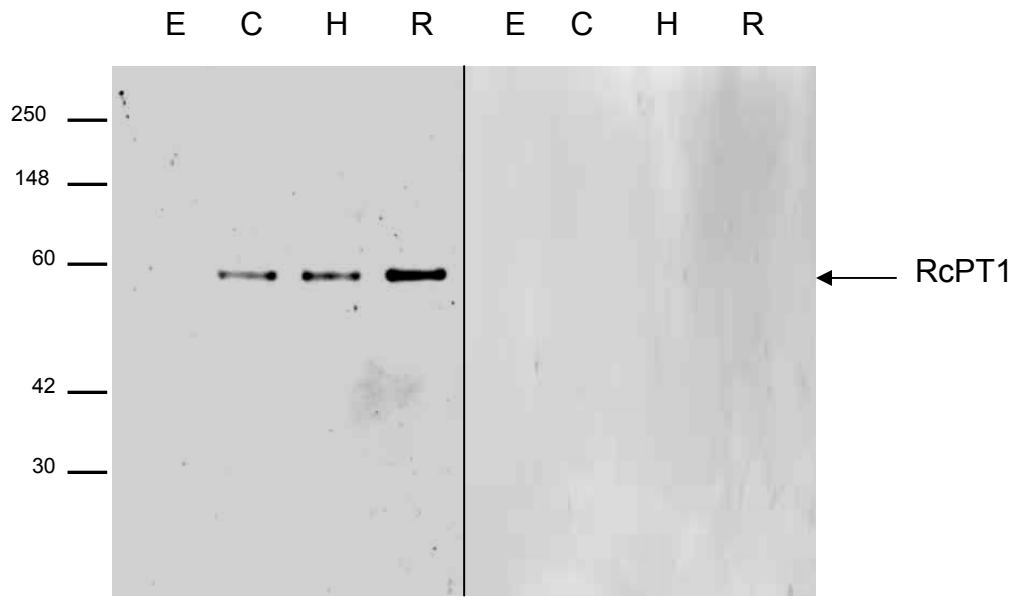


Figure 3.3.8: Accumulation of RcPT1 protein in 7-day-old *Ricinus* seedlings grown under Pi deficient condition. Western blot of total proteins isolated from endosperm (E), cotyledons (C), hypocotyl (H) and roots (R). The antibodies react with a 59-kD protein (left panel). No signal was detected with pre-immune serum (right panel). 20 µg of protein was loaded on each lane.

3.3.7 RcPT1 transcript induction under phosphate starvation in plants

The expression of *RcPT1* mRNA in *Ricinus* plants grown either in the presence of 500µM Pi (+Pi) or no Pi (-Pi) was compared by Northern-blot analysis. Hybridization with specific probes prepared from 3´-UTR of *RcPT1* cDNA showed that *RcPT1* was expressed in roots, stems and leaves in plants grown in Pi deficiency, while no signal was detectable in plants grown in +Pi. The accumulation of *RcPT1* in roots was higher than that in stems and leaves.

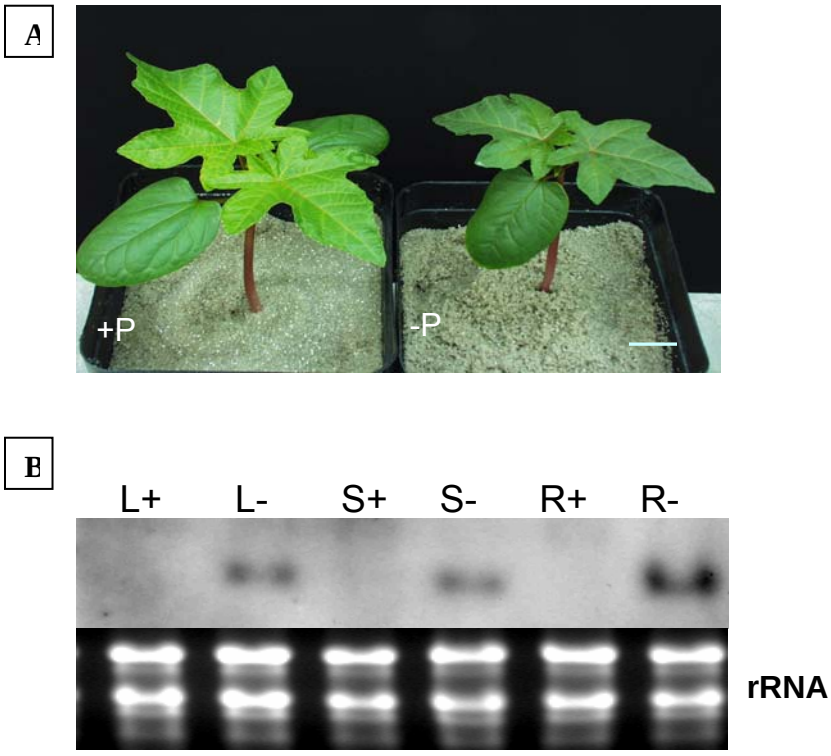


Figure 3.3.9: (A) 22 day-old *Ricinus* plants. (B) Expression of RcPT1 transcripts under Pi starvation. Northern blot analysis of total RNA isolated from leaves (L), stems (S), roots (R) of 22 days *Ricinus* plants grown in either Pi-deficient or Pi sufficient conditions. 10 µg of total RNA was subjected to Northern blot analysis. Gel pictures showing uniform loading of RNA. (Scale bar = 2 cm).

3.3.8 *In Situ* Hybridization of *RcPT1*

To gain further insight into the spatial patterns of *RcPT1* expression within the seedling and plant, *in situ* hybridization was performed with sense and anti-sense Dig-labelled RNA 3´-UTR of *RcPT1* (Figure 3.3.10).

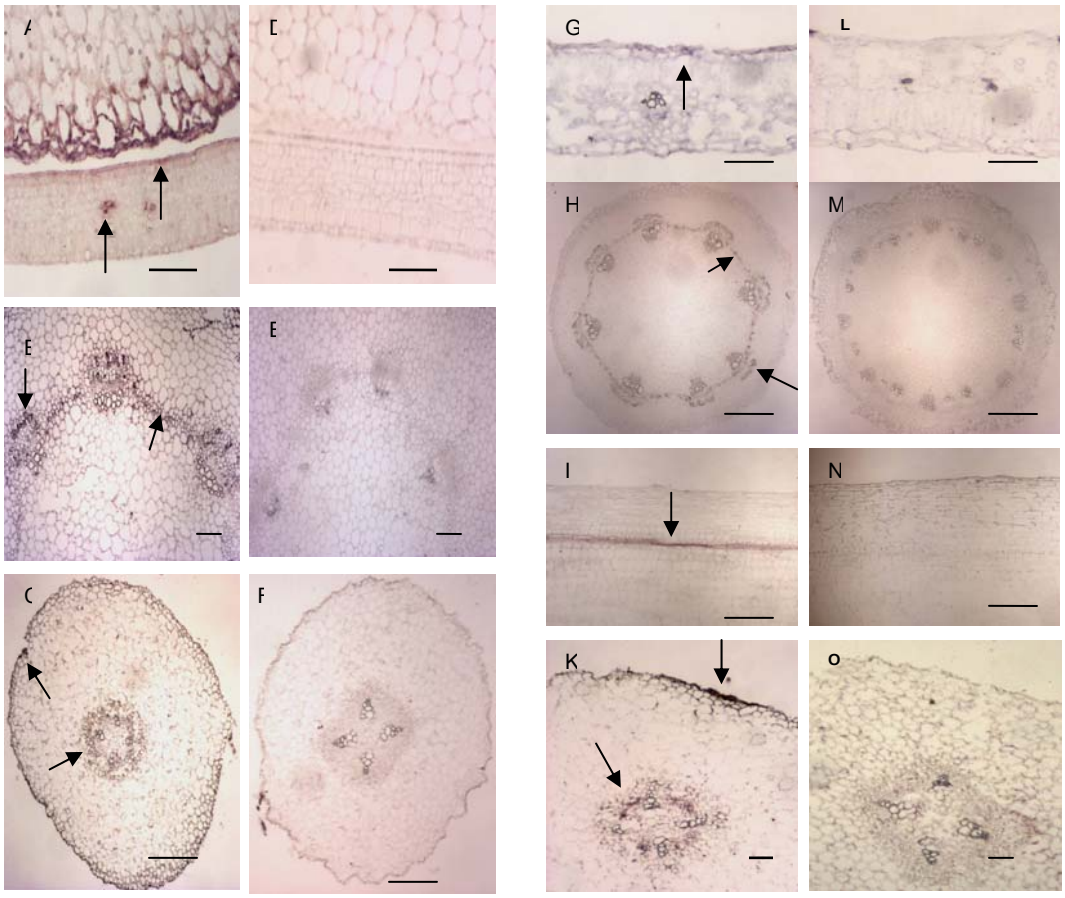


Figure 3.3.10: *In situ* localization of *RcPT1* in 6-day-old *Ricinus* seedlings (A-F) and plants at 22 days (G-O); A and D: sections of endosperm and cotyledon, B and E: sections of hypocotyl, C and F: transverse section of root, G and L: young leaf sections, H and M: transverse section of stem, I and N: longitudinal section of stem, K and O: transverse section of root. A, B, C, G, H, I, K. Sections hybridized with 3´-UTR of *RcPT1* Dig-labelled RNA probes anti-sense. D, E, F, L, M, N, O Sections hybridized with 3´-UTR Dig-labelled RNA probes sense. Arrows indicate the obtained signal.

Hybridization with anti-sense probe revealed that *RcPT1* transcripts accumulated in the endosperm, lower epidermis and cotyledon minor veins (Figure 3.3.10 A), phloem of the hypocotyl (Figure 3.3.10 B) and root epidermis and steles (Figure 3.3.10 C). No hybridization signal was observed in the section incubated with sense probe (Figure 3.3.10 D, E, F). In 22 day-old *Ricinus* plant, high accumulation of *RcPT1* mRNA was observed in the upper epidermis and minor vein of leaves (Figure 3.3.10 G). In addition the presence of *RcPT1* was also detected in the stem bundles (Figure 3.3.10 H, I) and in epidermis and stele of roots (Figure 3.3.10 K). No hybridization signal was observed in the section incubated with sense probes (Figure 3.3.10 L, M, N, O).

3.3.9 Immunolocalization of the RcPT1 protein

Immunolocalization of RcPT1 was performed to access the cellular location of the protein. The sections of 6-day-old seedlings were hybridized with the RcPT1 antibody or with pre-immune serum. Immunolocalization of the protein was performed in endosperm, cotyledons, hypocotyl and roots of seedlings at 6 days. A strong signal was observed in lower epidermis of cotyledons and adjacent area of endosperm (Figure 3.3.11 A), and the bundles of the hypocotyl (Figure 3.3.11). In longitudinal section of the root the signal was detected at the root tip, rhizodermis and stele (Figure 3.3.11 C). No signal was detected in section incubated with pre-immune serum. The results are evidence for a preferential accumulation of the protein in the same cell as its mRNA (Figure 3.3.11).

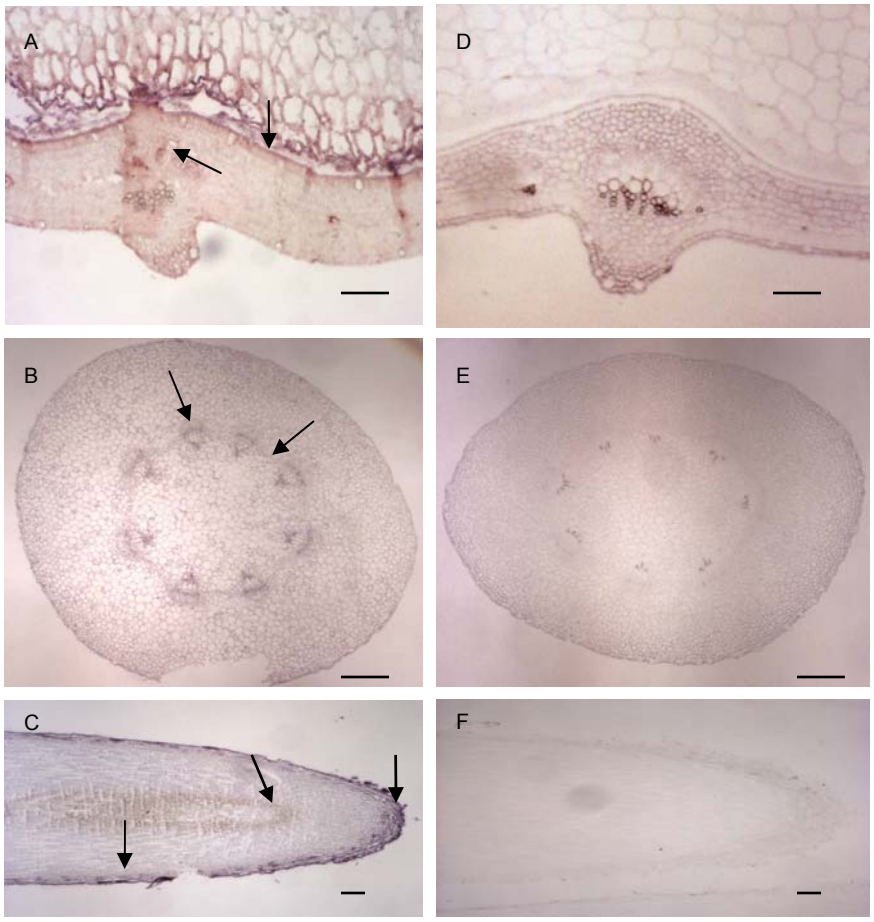


Figure 3.3.11: Immunolocalization of RcPT1 in transverse tissue sections prepared from 6-day-old *Ricinus* seedlings. A: sections of endosperm and cotyledon, B: sections of hypocotyl, C: longitudinal section of root. Sections A, B and C were probed with the affinity purified antibodies; sections D, E and F were probed with the pre-immune serum.

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3.4 Acid phosphatases

Plant acid phosphatases (APase) are involved in the phosphate metabolism of germination and development of plants. During germination, activities of APase increased to release the storage materials for the growth of seedlings (Biswas *et al*., 1991). In addition, APase appear to function in response to phosphate starvation in plants; both extracellular and intracellular, and are induced under Pi deficiency (Duff *et al*., 1994).

3.4.1 Cloning of *RcPS1*, a novel acid phosphatase and computational sequence analysis

In order to obtain a cDNA fragment of *RcPS1* from *Ricinus* seedling by RT-PCR, degenerated primers were designed based on the conserved region of the amino acid sequence of novel acid phosphatase genes from *Lycopersicon esculentum* (accession number AAG40473) and *Arabidopsis thaliana* (accession number AAM63155).
A degenerated forward primer *APRcF1* (5´-GTD TTY GAT TTY GAT AAD ACW ATY AT-3´) and a degenerated reverse primer *APRcR1* (5´-GGD CAD AAA TCW CCA ATW CCA T-3´) were deduced from the sequence of Motif 1 and Motif 2 of acid phosphatase proteins, respectively (Figure 3.4.1A). Initially, following optimal reaction conditions for successful PCR were identified.

1 cycle		94°C	120 sec
30 cycles	{	4°C	50 sec
		55°C	40 sec
		72°C	60 sec
Followed by		72°C	10 min

The obtained PCR product of approximately 560 bp was ligated in pGEM®-T Easy vector and sequenced. The amino acid sequence analysis indicated high similarity with the other acid phosphatase genes (Figure 3.4.1A). Based on the sequence of the initial PCR fragment sequence, gene-specific primers were designed.
The full length cDNA of RcPS1 was cloned by RLM-RACE (2.2.8).

3′ RLM-RACE was performed with *APRcF4* and *APRcF5* which are compatible with 3′ RACE outer primer and 3′ RACE inner primer, respectively; a PCR product of approximately 400 bp was obtained. While 5′ RLM-RACE was performed with *APRcR2* and *APRcR3* which are compatible with 5′ RACE outer primer and 5′ RACE inner primer, respectively; a PCR product of approximately 540 bp was obtained. After obtaining sequence information of the amplified products from 5′ and 3′ RLM-RACE, a pair of gene-specific primers *AP-RcF1* and *AP-RcR1* were deduced and used to amplify the full-length *RcPS1* cDNA.

RcPS1 cDNA is 1116bp long and contains a 747 bp open reading frame (ORF) encoding a peptide of 248 amino acids (27.5 kD). The open reading frame of *RcPS1* is flanked with 80 bp of untranslated sequence at the 5′end and 286 bp of untranslated sequence including poly (A^+) tail at the 3′end. Hydropathy plots of the deduced amino acid sequence shows that RcPS1 is a soluble protein (Figure 3.4.1).

A homology search of amino acid sequence with RcPS1 and the other phosphatase clones revealed that *RcPS1* shares 74%, 53%, 57% amino acid identity with phosphatases identified in *Lycopersicon esculentum* (AAG40473), *Arabidopsis thaliana* (AAM63155), and *Oryza sativa* (NP_916687), respectively. The comparison of RcPS1 with phosphatases from prokaryotes and eukaryotes suggested the presence of two highly conserved motifs, motif 1 “DFDXT” and motif 2 “GDGXXD”, which are the member of haloacid dehalogenase and DDDD superfamilies of enzymes catalyzing a diverse number of hydrolytic and phosphotransferase reactions (Thaller *et al*., 1998; Aravind *et al*., 1998). Collet *et al*., 1998 suggested that the first Asp in the motif “DFDXT” is phosphorylated during phosphate transfer reaction. The amino acid sequence of motif 1 “DFDXT” is conserved in phosphomutases and phosphatases. The motif 2 “GDGXXD” is mostly found in phosphatases rather than in phosphomutases.

Figure 3.4.1: (for figure in the next page) Alignment of protein sequences from various species and hydropathy profile of RcPS1. (A) Alignment of deduced amino acid sequence of RcPS1 with that of LePS2 (*Lycopersicon esculentum*), AAG40473; *O.sat* (*Oryza sativa*) NP_916687 ; *A.thal* (*Arabidopsis thaliana*) AAM63155. Identical amino acids are shaded in blue, similar amino acids are shaded in red. Two conserved motifs are indicated by asterisk. (B) Hydropathy profile of RcPS1 protein. Hydropathy values for a window of 7 amino acids were calculated by the method of Kyte and Dootline (Kyte *et al*., 1982).

A

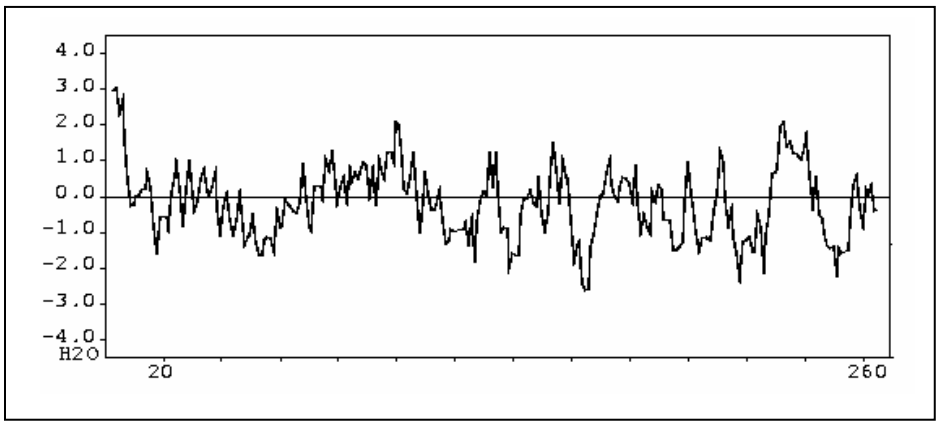
```
                          Motif 1
       1                                                                  70
RcPS1  ~~~~~~~~MA GILVVFDFDK TIVDVDSDNW VIDELGFTDL FNELLPTMPW NSLMDRMMKE LHSQGKTIED
LePS2  ~~~~~~~~MA GIVVVFDFDK TIIEVDSDNW VVDELGATDL FNQLLPTMPW NSLMDRMMKE LHTQGKTIQD
 Arab  MAYNSNNNNN NIVVVFDFDK TIIDVDSDNW VIDELGFTDL FNQLLPTMPW NTLMDRMMKE LHDQGKTIEE
 rice  ~~~~~~~~MA GIVVVFDFDK TIIDVDSDNW VVDGLGATEE FERLLPTMPW NTLMDTMMGE LHASGKSLAD

       71                                                                 140
RcPS1  MVEVLKRIPI HPRVIPAIKS AHALGCELRI VSDANLFFIE AILKHLGLRD YFSEINTNPG FVD.DEGKLR
LePS2  IEEVLKRVPI HPRIVPAIKS AHALGCDLRV ISDANVFFIE TILKHLGIRD CFSEINTNPG YVD.GEGRLR
 Arab  IKQVLRTIPI HPRVVPAIKS AHDLGCELRI VSDANMFFIE TIVEHLGISE LFSEINSNPG YVD.ERGTLK
 rice  VAGVLRSAPL DPRVVAAIKA CYGLGCDLRI LSDANRFFID TILDHHGLTG YFSEINTNPS AVDAATGRLR
                                                    Motif 2
       141                                                        210
RcPS1  IFPYHDFTQS SHGCS..LCP PNMCKGHIIE RIQSSISKED KSKKIIYLGD GIGDFCPSLK LTEADYLMPR
LePS2  ILPYVDFQKS PHSCN..LCP PNMCKGMIVE RIQ...AKEG K.KRMIYLGD GIGDFCPSLK LREADFVMPR
 Arab  ISPYHDFTKS PHSCSCGTCP PNMCKGLIIE RIQQSLAKEG K.KKMIYLGD GAGDYCPSLK LNTEDYVMPR
 rice  IAPYHDFHAG PHGCGLGICP PNMCKGQVLD RIRASAGAAG ..KRVIYLGD GRGDYCPSLR LGRDDFMMPR

       211                                                                280
RcPS1  KNFPVWDLIC SNPMVIKAEI HEWIDGEELE RVLIEIIDGI CLEEINGSSD LFSS.DCKLQ TVPIA...A.
LePS2  KDFPAWNLIN KNRTLVKAGV HEWTNGKELE HILLQWINTI NIEE...SQL LSME.NCKFQ TKHNAAHGA.
 Arab  KNFPVWDLIS QNPMLIKAVI REWTDGQSME MILIGTIEEI RLEEEKEKML TSAENNCKMQ TISIGINNVH
 rice  RGFPVWELIC EDPSLLHAEV HSWADGAEME ETLLRLVGRV LLEERNLPPL .....DCKLE SLP....AVA

       281          295
RcPS1  ....LPQAVP VPQL~
LePS2  ....LPRPLP VPY~~
 Arab  HEPILPRALR VSQSS
 rice  VQDGMPMTLR IKN~~
```

B



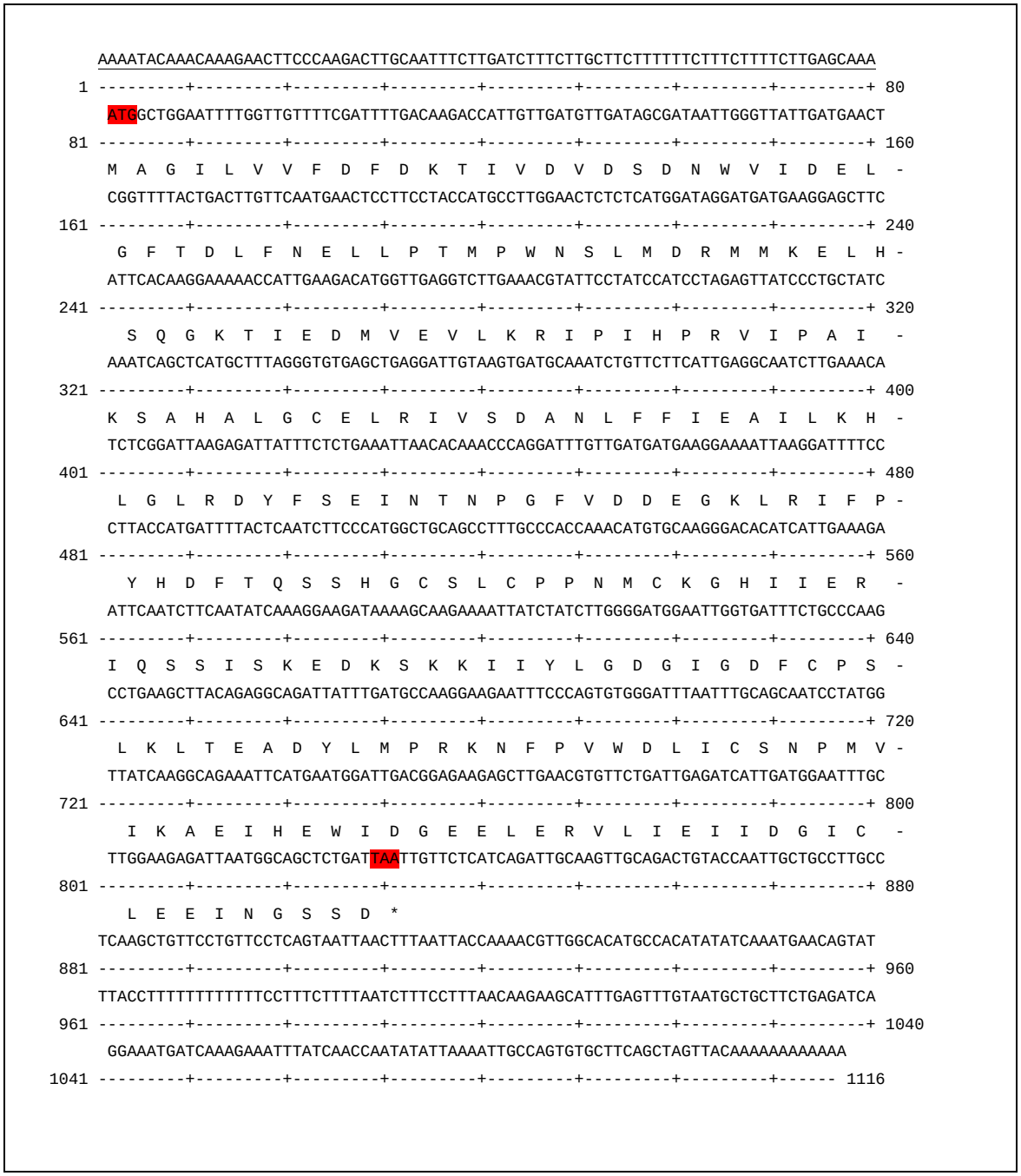


Figure 3.4.2: The nucleotide and amino acid sequence of the *RcPS1* full-length cDNA. The 5´-untranslated region of 80 nucleotides is underlined. Start codon and stop codon are coloured in red.

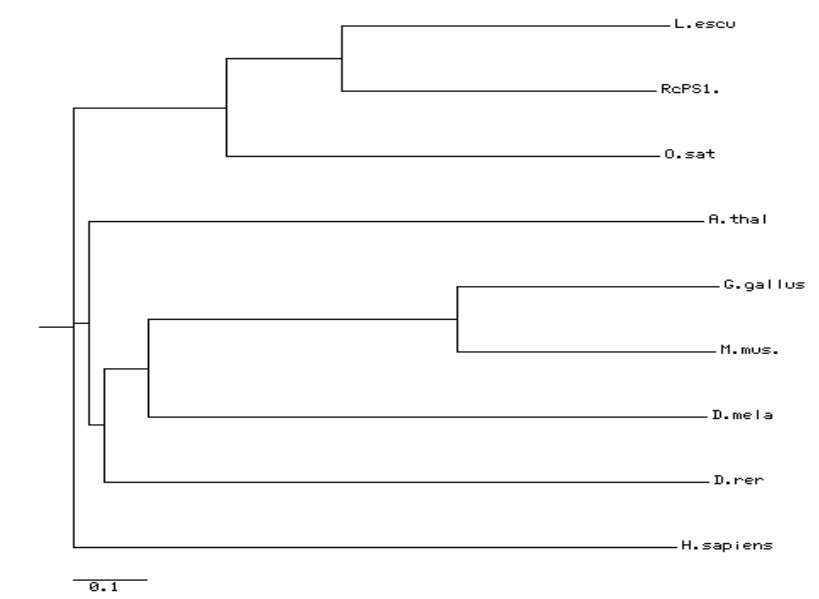


Figure 3.4.3: Phylodendrogram showing the relationship between *RcPS1* and related amino acid sequences of phosphatases from *L.esc* (*Lycopersicon esculentum*), accession number AAG40473; *O.sat* (*Oryza sativa*) NP_916687 ; *A.thal* (*Arabidopsis thaliana*) AAM63155; *G.gallus* (*Gallus gallus*) NP_990176; *M.mus* (*Mus musculus*) CAD29804; *D.rer* (*Danio rerio*) XP_687716 ; *D.mela* (*Drosophila melanogaster*) AAF48992; *H.sapiens* (*Homo sapiens*) AAH22324. Sequence divergences are related to branch lengths, and can be estimated relative to the 0.1 bar shown.

To estimate the phylogenetic relationship between *RcPS1* and the other phosphatases genes, we used Clustal X alignment to determine the cluster. The result revealed the existence of RcPS1, *L.esc* and *O.sat* in one group. It is in agreement with the result of high identity between RcPS1 and *L.esc* (*Lycopersicon esculentum*), AAG40473. *A.thal* falls outside of this group although it has virtually identical amino acid sequences, but varied in its coding.

3.4.2 Genomic organization of *RcPS1* gene

Southern blot was performed to estimate the number of sequences present in the *Ricinus* genome that are homologous to *RcPS1* cDNA. The full length of *RcPS1* cDNA was used for synthesis of Dig-labelled RNA probe. The blot was washed at a high stringency and showed the presence of three bands. *Hind III* and *EcoRI* have a restriction site in the *RcPS1* cDNA, whereas *BamHI*, *XbaI* and *EcoRV* have no restriction sites in the *RcPS1* cDNA. The result shows that *RcPS1* gene is belonging to a small gene family.

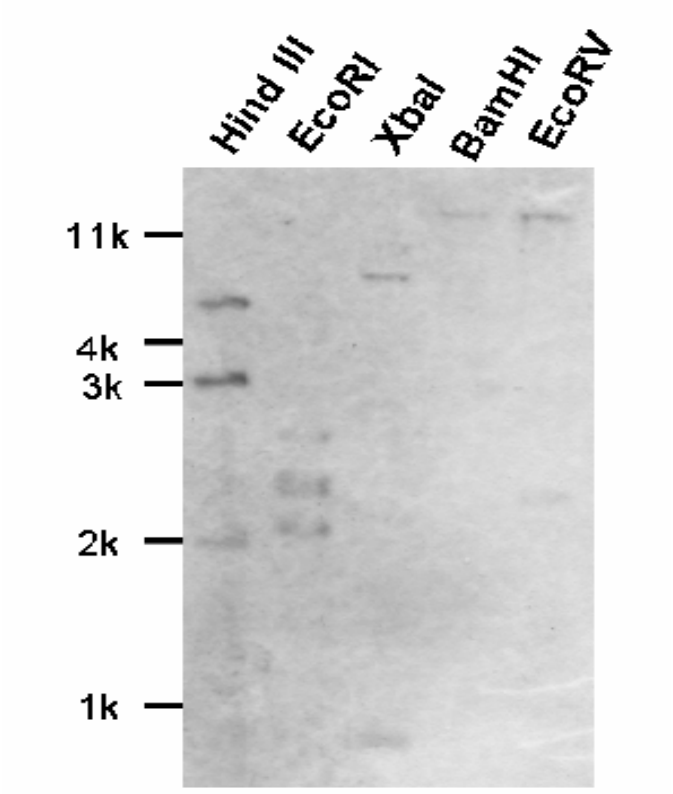


Figure 3.4.4: Southern blot hybridization of *RcPS1* cDNA to genomic DNA digested with the restriction enzymes. The position of DNA marker fragments and their lengths in kilobases are indicated at left.

3.4.3 Expression of recombinant *RcPS1* in *E.coli*

The full length RcPS1 cDNA was cloned into the pET28b expression vector (Novagen). This expression system allowed the production of recombinant proteins in *E.coli* regulated by isopropylthio-ß-galactoside (IPTG) and the purification of recombinant protein via His tag and metal affinity column.

After 2 hours induction with IPTG at 30°C, bacteria expressed RcPT1 to a high level. Purification of protein was performed through several steps with different concentrations of imidazol applied to the nickel activated affinity column to guarantee maximum purity of the eluted protein.

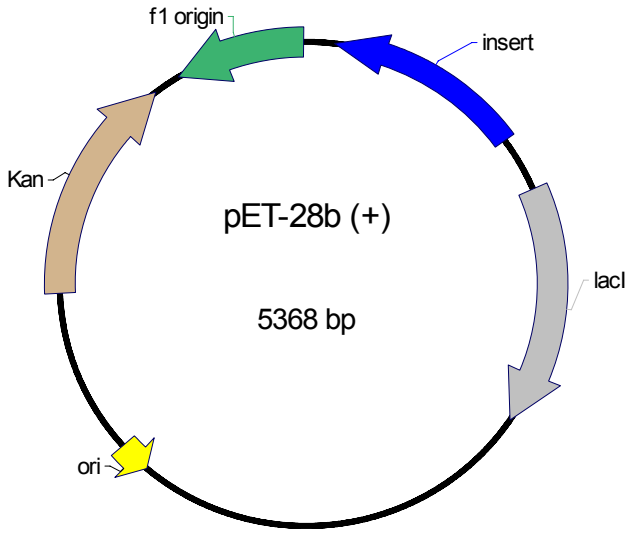


Figure 3.4.5: Cloning of *RcPS1* cDNA into expression vector pET28b.

The purified proteins were examined by SDS-PAGE. It showed due to the His tag (approximately of 3 kD) a band at approximately 28 kD, larger than that expected from amino acid sequence. Phosphatase activity was analyzed by using *p*-nitrophenyl phosphate as a substrate. Although activity was not high, it showed significant phosphatase activity, 6.19 fold higher than purified protein from control, empty vector.

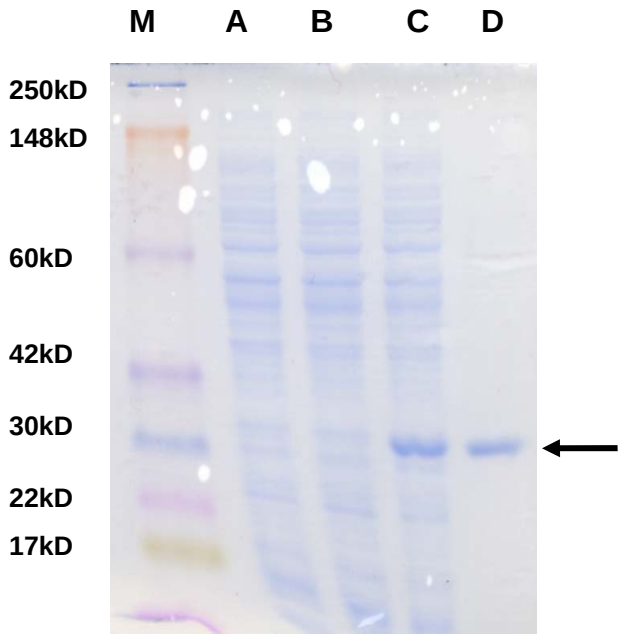


Figure 3.4.6: SDS-PAGE analysis of expression and purification of RcPS1 in *E.coli* BL21 cells by using pET28a vector. M, Marker; A:BL21; B: vector; C: crude extract; D: recombinant RcPS1 protein. Arrow indicates the expected size of recombinant RcPS1 protein.

3.4.4 Expression pattern of *RcPS1* in seedling organs during germination

It is suggested that acid phosphate activity is increasing during germination and plays an important role in seedling growth. To determine the expression pattern of *RcPS1* gene in different plant organs in the early stage of germination, RNA was extracted from endosperm, cotyledons, hypocotyl and roots from 4 to 7-day-old *Ricinus* seedlings. In Northern blot, Dig-labelled RNA probes from the 3´-UTR of *RcPS1* were synthesized.

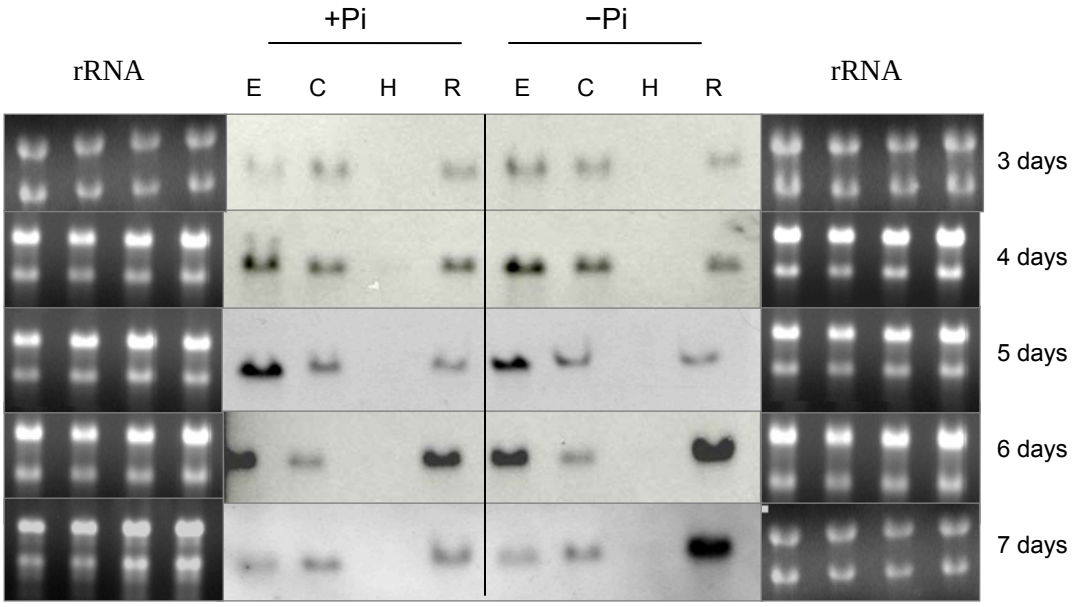


Figure 3.4.7: Northern blot analysis of acid phosphatase *RcPS1* mRNAs in different organs at various stages of germination. Total RNA from endosperm (E); cotyledons (C); hypocotyl (H); and roots (R) of *Ricinus* seedlings grown in either 500 µM Pi (+Pi) or no Pi (-Pi) were hybridized to anti-sense probe corresponding to 3´-UTR of *RcPS1* cDNA. 10 µg of total RNA was subjected to Northern blot analysis. Gel pictures in the left and the right side of blot show uniform loading of RNA.

Similar expression levels of *RcPS1* were obtained from seedlings grown with Pi or without Pi. The result demonstrated that the expression of *RcPS1* was independent from the exogenous Pi supply. However, the expression of *RcPS1* varied in different organs of the seedling, because it accumulated in cotyledon, strongly accumulated in endosperm and root at day 6, and no signal was found in hypocotyl. At day 7, the expression of *RcPS1* decreased in endosperm probably due to senescence progression and collapse of the endosperm. In the root of seedlings grown without Pi, the expression of *RcPS1* was increased compared to expression under condition with Pi.

3.4.5 *RcPS1* transcript induction under phosphate starvation in plant

To determine the tissue specificity of the expression of RcPS1, RNA was extracted from different organs of 22 days *Ricinus* plants grown either with or without Pi. The expression of *RcPS1* was found in all parts of plants, which were subjected to the condition without Pi, and no signal was detectable in presence of Pi. *RcPS1* was expressed strongly in stems

These results indicated that expression of *RcPS1* was regulated by exogenous Pi supply in *Ricinus* plants, suppressing *RcPS1* expression when Pi uptake reached the Pi demand of the plants. *RcPS1* was expressed in all parts of the plant under Pi deficiency, it suggests its role in enhancing Pi availability and possible recycling of organic Pi in plants.

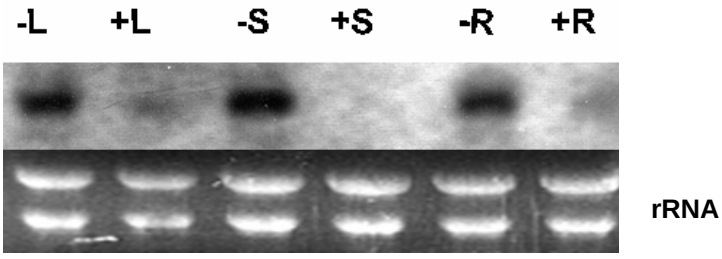


Figure 3.4.8: Expression of RcPS1 transcripts under Pi starvation. Northern blot analysis of phosphate transporter *RcPS1* gene in Ricinus 22 days old plants grown in sand in presence of Pi (+) or without Pi (-); leaves (L), young stems (S), roots (R). 10 µg of total RNA was subjected to Northern blot analysis. Gel pictures showing uniform loading of RNA

3.4.6 Localization of *RcPS1* transcript in cotyledon and leaf

In order to understand the expression of *RcPS1* at cellular levels, its transcript abundance in endosperm, cotyledons and roots of 6-day-old *Ricinus* seedlings and leaves, stems and roots of 22 days old plants were analyzed by i*n situ* hybridization.

The results showed that *RcPS1* was found in the endosperm (Figure 3.4.9 A), lower and upper epidermis of cotyledons, and the phloem of cotyledons of 6-day-old seedlings (Figure 3.4.9 B). Expression of *RcPS1* was also observed phloem and stele of roots. No hybridization signal was observed in the section incubated with sense probe (Figure 3.4.9 E, F, G, H and Figure 3.4.10 D, E, F). High accumulation of mRNA was also obtained in upper epidermis of leaves, stem vasculature; epidermis and stele of roots from 22 days old *Ricinus* plants (Figure 3.4.11 A, B, C, D), while sections which were incubated with sense probe showed no signal (Figure 3.4.11 E, F, G, H).

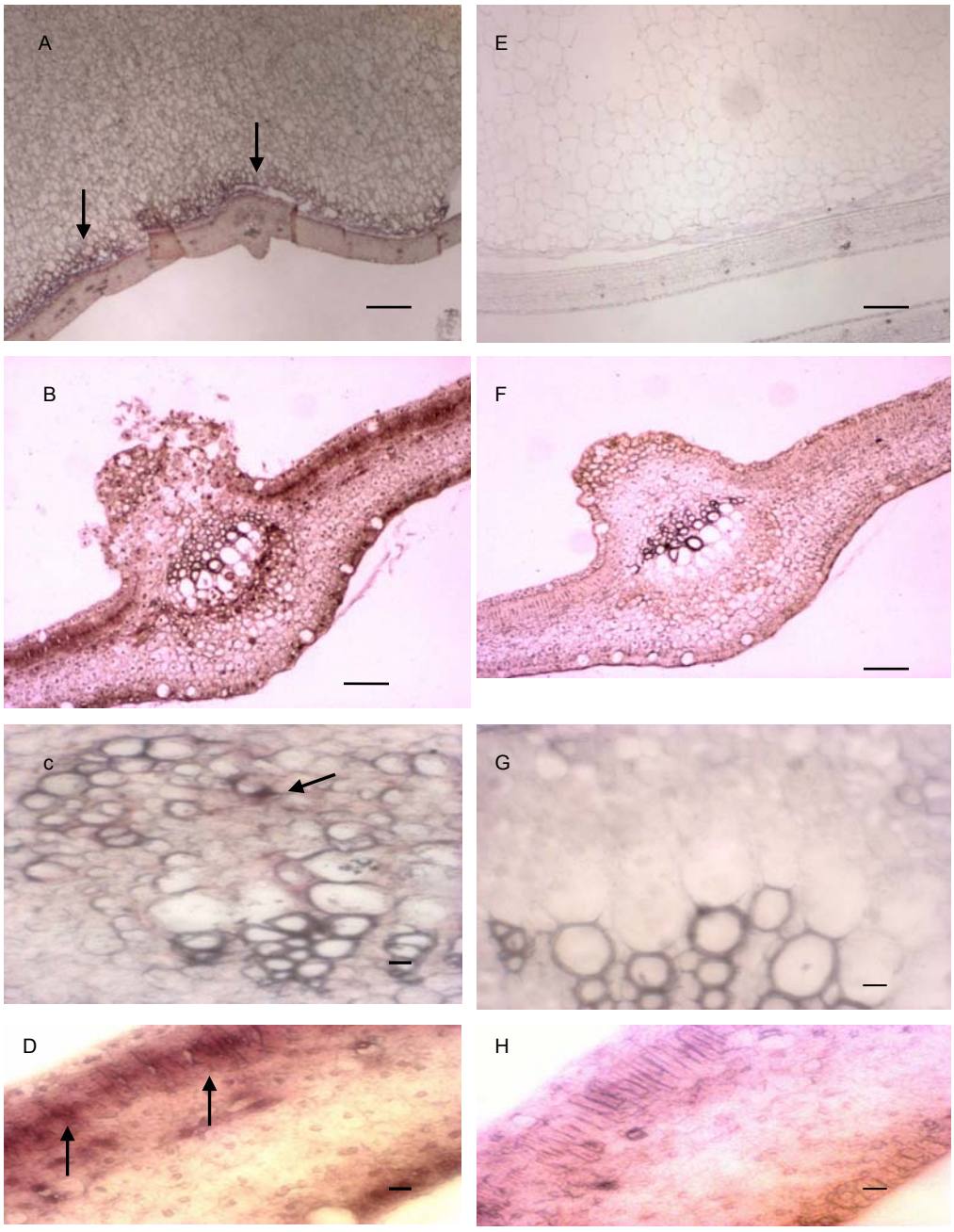


Figure 3.4.9: (for the figure in the previous page) *In situ* localization of *RcPS1* in 6-day-old *Ricinus* seedlings A and E: sections of endosperm and cotyledon, B and F: section of cotyledon, C and G: magnification of cotyledon section. Detection by hybridization with Dig-labelled RNA anti-sense probe. Sections A, B, C, and D: Detection by hybridization with Dig-labelled RNA anti-sense probe. Sections E, F, G, and H: Detection by hybridization with Dig-labelled RNA sense probe. Arrows indicate the obtained signal.

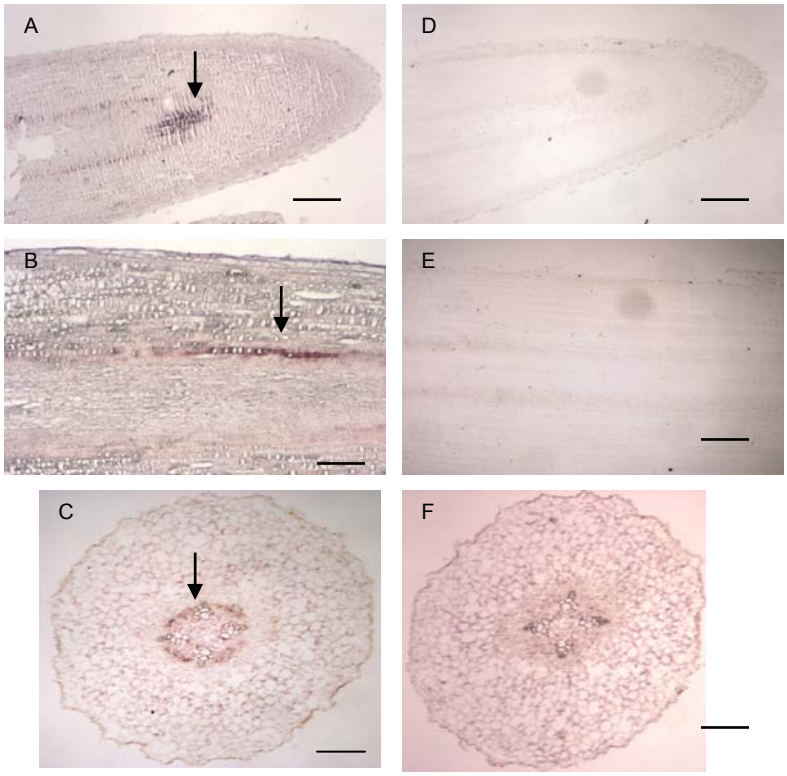


Figure 3.4.10: *In situ* localization of *RcPS1* in A-D: longitudinal section of root, B and E: High magnification of transverse section of root, C and F: transverse section of root. A, B, C Section hybridized with *RcPS1* anti-sense probe, D, E, F Section hybridized with *RcPS1* sense probe. Arrows indicate the obtained signal.

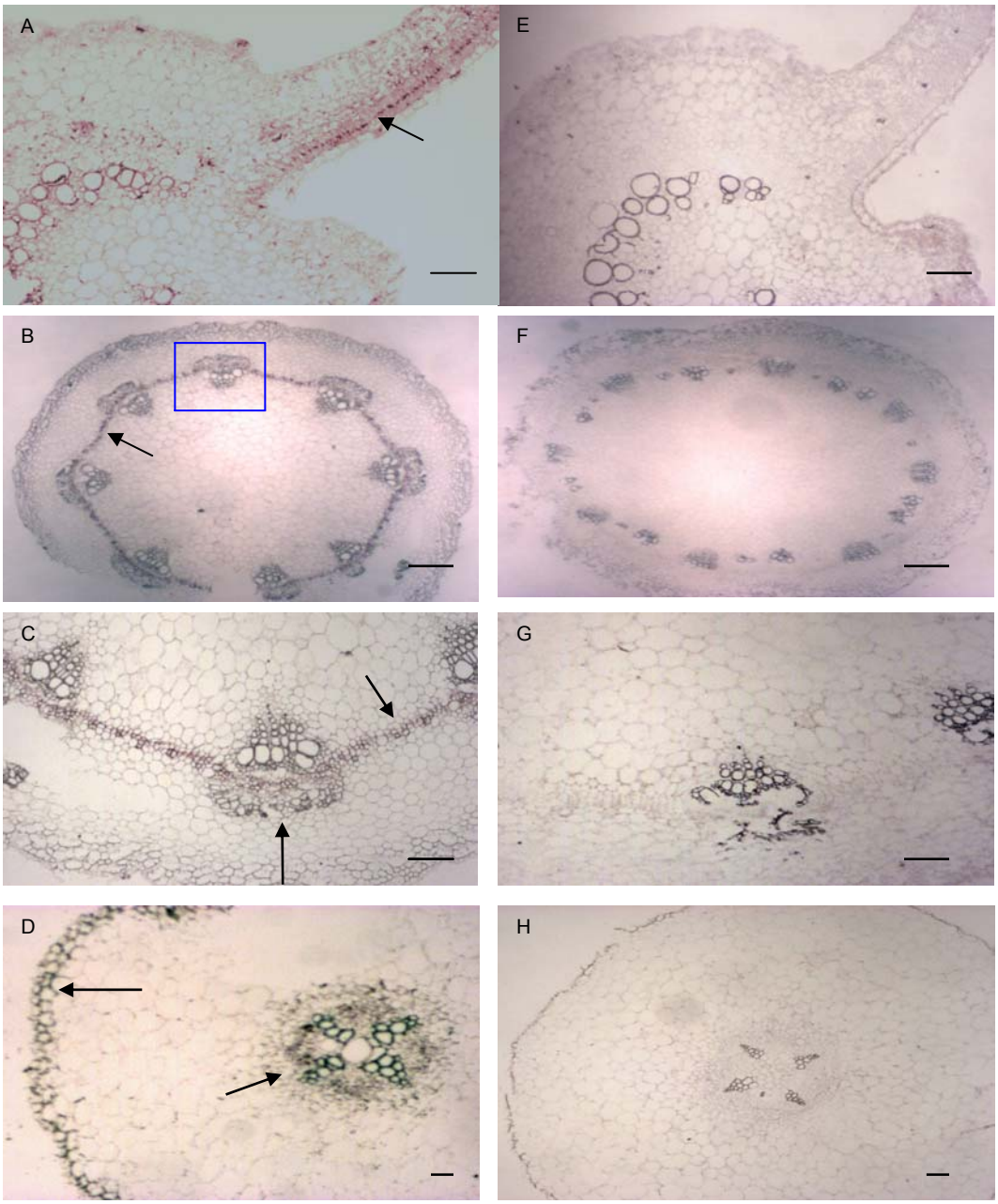


Figure 3.4.11: *In situ* hybridization on sections of leaves, stems and roots from the *Ricinus* plants at 22 days age. G and L: section from young leaf, H and M: transverse section of stem, I and N: longitudinal section of stem, K and O: transverse section of root. A, B, C, G, H, I, K. Sections hybridized with 3´-UTR of *RcPS1* Dig- labelled RNA anti-sense probes. D, E, F, L, M, N, O Sections hybridized with 3´- UTR Dig-labelled RNA sense probes. Arrows indicate the obtained signal.

4 Discussion

4.1 Phosphate homeostasis in *Ricinus* seedlings

Pi is an important element for plant growth and development. However, the availability of Pi in natural ecosystem is often not sufficient for growth, thus plants need to develop an adaptive system for changing morphological, biochemical and symbiotic strategies in order to acquire Pi for their life. In another word, plants need to develop homeostatic mechanisms to regulate their Pi status. Despite many studies about the involvement of Pi in plant growth and development, little is known about the specific transport systems that are essential for the Pi uptake and for internal Pi redistribution within plants. It is suggested that the levels of phosphates, as sum of inorganic and organic, in the cytoplasm are maintained constant, whereas Pi levels in vacuoles are varied under Pi deficiency (Mimura *et al*., 1992). This indicated that cytoplasm and vacuolar function as specific Pi storage compartments in plants cells; vacuoles act as “Pi pool” buffer between cytoplamic and external Pi.

It has been reported that there are three distinct transport systems that may be involved in Pi translocation within the plant: i) secretion of Pi into the xylem in the root; ii) loading of leaf cells with Pi from the vascular system; and iii) Pi cycling in the phloem within the plant during senescence or from storage organ (leaves) to roots during Pi starvation (Bucher *et al*., 2001). The main purpose of these experiments is to clarify the translocation of Pi along the seedlings via phloem and via xylem. For this purpose ^{32}P was sequentially applied to the cotyledons and to the roots.

4.1.1 Phosphate uptake via cotyledons

In general, the radio isotope ^{32}P has been used to study the movement of Pi in plants. The re-translocation of Pi from old leaves to young leaves of barley under Pi deficient condition was detected by using ^{32}P. It is supposed that the movement of Pi in leaves occurs via the phloem and it may involve symplastic and apoplastic pathways (Mimura *et al*.,1994).

In this study, ^{32}P as KH_2PO_4 was used in the experiments for Pi movement in *Ricinus* seedlings. The translocation of Pi was detected from the cotyledons to the hypocotyl, particular to its apical hook near the cotyledons (Figure 3.1.2). ^{32}P accumulation decreased from the hypocotyl towards the roots. After 20 min exposure to ^{32}P, a high amount of radio tracer was seen in the cotyledon minor veins, the major site of phloem loading (Figure 3.1.1). These results suggested that Pi is loaded into the phloem. In previous studies in our group it was shown that the phosphate levels in endosperm, cotyledons and in the sieve tube-sap increased when the apoplast was equilibrated with different concentrations of phosphate (Bauer-Ruckdeschel, 2001). In contrast, for the transport of nitrate and ammonium into the phloem, Schobert and Komor, (1992) reported the presence of restricted loading of nitrate into the phloem and ammonium was absolutely not taken up.

The phosphate exported into the phloem was partly re-circulated in the xylem though to a minor extent. The balance between uptake and loss is critical for plant growth, thus the efflux of Pi by passive leakage and the backflow through the uptake systems is important. Pi taken up by *Ricinus* cotyledons moves through the hypocotyl and partially re-circulates in the xylem or leaks out from the roots. Therefore reducing the efflux could be as important for the plant as increasing the affinity of the uptake mechanism. It is supposed that the Pi homeostasis is controlled by Pi-efflux when external Pi is high, increase of Pi-efflux almost compensates for the higher Pi-influx (Elliott *et al*., 1984; Cogliatti *et al*., 1990).

4.1.2 Phosphate uptake via roots

Movement of Pi from root to shoot occurs via xylem. Plasma membranes of root epidermal and cortical cells provide the boundary between the apoplasm and symplasm of roots involved in nutrient uptake. In the symplastic path, nutrient ions in cytoplasm can move radially through the stele via plasmodesmata connections without encountering further membrane barriers (Clarkson, 1993). However, for the symplasmic movement to occur, a gradient in concentration between outside and inside would be expected.

The movement of nutrient ions across plasma membrane of root epidermal and cortical cells is considered as a significant step in the uptake of the nutrient into the plant. This

movement process involves the transport systems which are either a high affinity system active at low external concentrations or a low affinity system active at high external Pi concentrations. This suggests that plasma membrane functions as mediator and regulates nutrient uptake. Uptake of Pi into the root symplasm involves transport from the apoplasm. However, due to the large concentration difference between apoplasm and cytoplasm, and the net negative charge on the inside of the plasma membrane, a strong electrochemical gradient needs to be overcome for the transport of Pi anion into root cells (Mimura, 1999).

In this work, the results of ^{32}P uptake into the roots of *Ricinus* seedlings showed that a high amount of radiotracer accumulated in the cotyledons after one hour exposure to the roots to ^{32}P. It is implicated that Pi was transported first via xylem to the cotyledons through the hypocotyl (Figure 3.1.4). Jeschke *et al*. (1997) indicated that the Pi absorbed from external Pi environment by the roots is transported via the xylem to young leaves. Under low external Pi supply, there is significant re-translocation of Pi in the phloem from older leaves to the growing shoots and subsequently to the roots. In Pi-deficient plants the restricted supply of Pi to the shoots from the root via the xylem is supplemented by increased mobilization of stored Pi in the older leaves and re-translocation to both younger leaves and growing roots. This process involves the depletion of Pi stores and the breakdown of organic P in the older leaves.

After incubation of ^{32}P to the root, radioactivity was found in the main and minor veins of cotyledons as shown in Figure 3.1.3. Thus Pi mobilization occurred via re-translocation from the cotyledons to the roots via phloem. It is suggested that the Pi homeostasis is controlled by P-efflux when external Pi is high, increase of P-efflux almost compensates for the higher P-influx. In their experiment of Pi uptake in the roots of wheat plants, Cogliatti and Santa, (1990) showed that the P-efflux increased with increasing external concentration of Pi, from 28% of influx at 50 µM to 90% at 5 mM Pi. In the phloem phosphorus (P) is transported to a significant amount as organic P, while Pi is transported in the xylem (Bieleski, 1973).

It was found that during the early phase of germination of *Ricinus* seedlings no phytic acid accumulation in the isolated cotyledons was identified in the absence of exogenously supplied Pi or in the presence of 1 mM Pi in the incubation medium, whereas phytic acid was identified in the presence of 2 mM to 10 mM Pi (Organ *et al*.,

1988). Thus, another possibility for Pi storage when high Pi concentration in the cotyledons occur that may lead to phytic acid synthesis which is deposited in the vacuole of cotyledons as Pi storage pool. The obtained results from this study reveal that Pi transport through the hypocotyl is independent of the external Pi concentrations. The high accumulation of Pi in the hypocotyl hook, the elongation zone of the seedling, may be due to the high requirement of Pi in growing organs.

4.2 The involvement of phosphate transporter *RcPT1* and acid phosphatase *RcPS1* during germination and development of *Ricinus communis* L. plants

4.2.1 Cloning and molecular characterization of *RcPT1* cDNA

Phosphate transport in plants consists of high-affinity and low-affinity components. During the previous decade, a number of phosphate transporters which are involved in a high-affinity system and low-affinity system have been isolated from different plant species (Raghothama, 1999). Their functions have been recognized as being essential in the movement of Pi in plants, including the uptake of Pi and the internal redistribution of Pi within plants.

In this study, a full length cDNA clone of phosphate transporter, *RcPT1* was isolated from *Ricinus* seedlings grown in Pi starvation conditions. *RcPT1* is 1680 bp long and contains an open reading frame encoding a 530 amino acid polypeptide with a calculated molecular mass of 59 kD. The deduced amino acid sequence of RcPT1 showed significant high similarity to other known high-affinity Pi transporters, such as *Arabidopsis thaliana* (AtPT1, accession number Q96302), *Lycopersicon esculentum* (LePT1, accession number O22548), *Medicago truncatula* (MtPT1, accession number O22301), *Solanum tuberosum* (StPT1, accession number Q43650), *Oryza sativa* (OsPT11, accession number AN39052), *Catharanthus roseus* (PIT1, accession number O22055). These genes encoding high-affinity phosphate transporters and have been grouped into the *Pht1* family of proton-Pi co-transporters (Bucher *et al*., 2001), which are supposed to be energized by plasma membrane proton ATPase (Schachtman *et al*., 1998).

Hydrophobic analyses indicate that RcPT1 has 12 membrane-spanning domains with two separated groups of six interrupted by a large hydrophilic, charged domain of 61 amino acids. It is supposed that the N-terminal and C-terminal part of RcPT1 and long central loop are faced toward the inner membrane (Figure 4.2.1). This structure is shared by a number of other membrane transporters that belong to the major facilitator super family of protein responsible for transport of sugars, amino acid and ions (Saier and Reizer, 1991; Pao *et al*., 1998). Pi transporters isolated from plants and fungi have similar features, such as a peptide sequence containing 518-587 amino acids and utilization of a H^+ gradient to drive the symport process. Moreover, the structure and charge of membrane-spanning domains, and amino acid residues in the hydrophobic loops play important role in the transport of ion through the membrane (Smith *et al*., 2000).

Peptide sequence of RcPT1 shows putative phosphorylation sites and a glycosylation site, which have also been seen in the fungal phosphate transporters. Although it is supposed that Pi transporters isolated from plants and fungi belong to a closely related family, the similarity between the plant transporters was found to be higher than that between plant and fungal transporters (Muchhal *et al*., 1996).

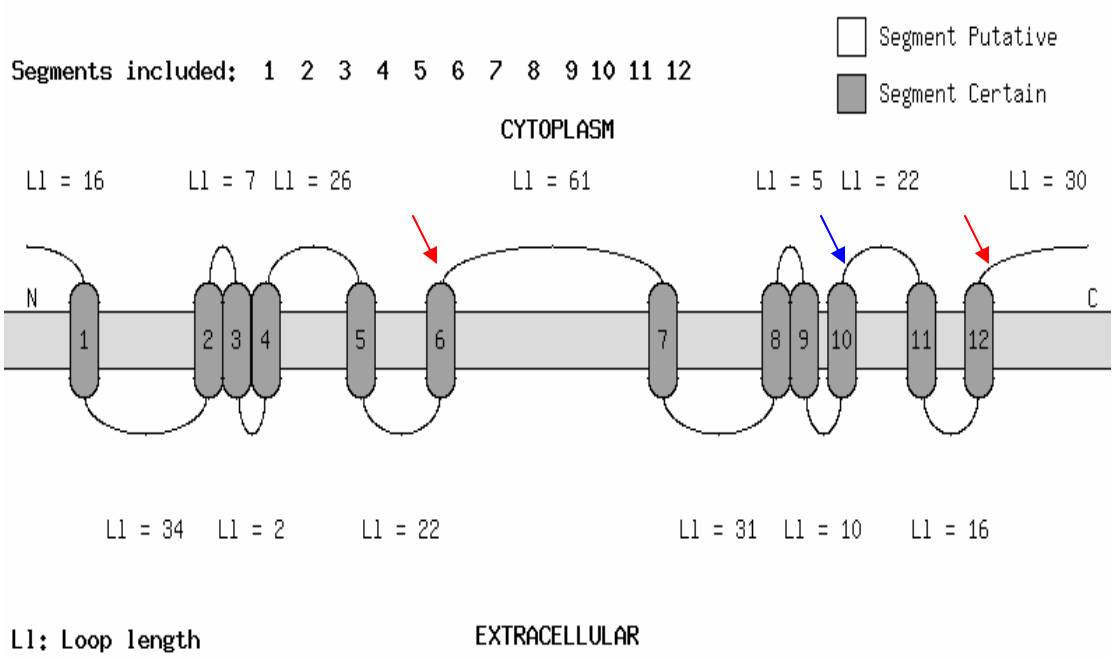


Figure 4.1.1: The topology of the high-affinity Pi transporter (RcPT1) from *Ricinus*, with 12 membrane-spanning domains arranged in a well defined six-loop-six configuration. The N- and the C- terminus and a long central loop containing 61 amino acids between membrane-spanning domains 6 and 7 are predicted to be intracellular. Red arrows indicate putative phosphorylation sites and blue arrow indicates the site for N-glycosylation.

Table 4.1.1: The beginning and the end of amino acids of each membrane-spanning segment in RcPT1 protein sequence are presented in the columns named “Begin” or “End”. The results were obtained by using TopPred 2 tool (http://bioweb.pasteur.fr/seqanal/tmp/toppred).

Helix	Begin	End	Helix	Begin	End
1	17	37	**7**	295	315
2	72	92	**8**	347	367
3	100	120	**9**	373	393
4	123	143	**10**	404	424
5	170	190	**11**	447	467
6	213	233	**12**	484	504

The similarities between the topology of phosphate transporters in various plant species can support the hypothesis that at least in some diploid plant genomes a redundancy of genes encoding phosphate transporters is critical to plant survival. Based on information of phosphate transporter peptide sequences, the structure and function of phosphate transporters can be further studied by using site-directed mutagenesis in order to change potentially important membrane-spanning domains (Smith, 2002).

The fact that plant phosphate transporters contain highly conserved sites for amino acid glycosylation and phosphorylation, indicates the existence of post-transcriptional control mechanisms. It is suggested that interactions between transporter and other proteins may play an important role in Pi uptake. These proteins have been characterized in PHO-regulon from yeast (Oshima *et al*., 1996; Ogawa *et al*., 2000) and were supposed to have the same function in plants. The study of the conserved sites would be required to get insight into the interaction between a number of proteins which are involved in regulating the activity of plant Pi transporters (Bucher *et al*., 2001).

Yeast (*Saccharomyces cerevisiae*) contains high and low-affinity Pi uptake systems, in which transcription of high-affinity phosphate transporter (*PHO84*) is controlled by different regulators of PHO-regulon based on the availability of Pi in the medium (Youshida *et al*., 1987; Johnston and Carlson, 1992). The structure-function relationship of plant's phosphate transporters can be examined by using the yeast system after site-directed mutagenesis to alter the potential important domains; the effects of these alterations on transport can be determined by heterologous expression. Various phosphate transporters from potato, *Arabidopsis*, tomato, *Catharanthus roseus* (Muchhal *et al*., 1996; Leggewie *et al*., 1997; Kai *et al*., 1997; Lu *et al*., 1997; Daram *et al*., 1998) have been used to complement yeast mutants lacking the high-affinity Pi transport mechanism. The functional analysis results in the high-affinity phosphate transporter yeast mutant *MB192*, which carries a mutation in the *PHO84* gene (BunYa *et al*., 1991), showing that the expression of *RcPT1* enhanced the ability of the RcPT1 transformants in restoration of Pi uptake capacity, thus these transformants grew faster than the yeast mutant *MB192* on low-phosphate medium (Figure 3.3.6). In the yeast mutant, a low intra-cellular Pi concentration would lead to synthesis of APase due to lacking in ability for Pi uptake. However, the restored Pi uptake transformant expressing *RcPT1* showed no acid phosphate activity because the transporter activity of RcPT1 protein that enhanced uptake of phosphate into the cells to a high intracellular concentration, thus

repressing APase formation. These results indicate that *RcPT1* really encodes a phosphate transporter.

4.2.2 Expression of phosphate transporter *RcPT1* in response to Pi starvation conditions

Pi is taken up by plant roots and transported into the xylem parenchyma and subsequently secreted into the xylem (apoplast) for long distance translocation to the above ground organs (Jeschke *et al*., 1997). An energy mediated co-transport process, driven by protons generated by plasma membrane H^+-ATPase, has been proposed as the mechanism of Pi uptake in plants. In general, plants store Pi in the vacuole to regulate Pi homeostasis. The cytoplasmic Pi concentration is maintained constant under varying levels of Pi supply by expending the vacuolar Pi, whereas the vacuolar Pi levels decrease significantly under Pi deficiency. Transport between the vacuole and cytosol take place via the tonoplast membrane and involves both influx and efflux (Mimura, 1995).

Although, little is known about the molecular mechanism of Pi efflux, studies using molecular probes derived from genes encoding plant phosphate transporters have proposed that transcriptional control is an important regulatory mechanism for the genes involved in Pi influx. Transcriptional regulation appears to be important for the expression of many plant transporters. The expression of *Arabidopsis* Pi transporters *Pht1;1* and *Pht1;2* increased greatly when roots were deprived of Pi and expression levels decreased immediately after re-supplying Pi to the plants (Smith *et al*., 1997a). Various genes encoding Pi transporters from potato (Leggewie *et al*., 1997), *Arabidopsis* (Mitsukawa *et al*., 1997a), tomato (Daram *et al*., 1998), *Medicago* (Liu *et al*., 1998b), barley (Smit *et al*., 1999), *Lupinus albus* (Liu *et al*., 2001), were shown to be regulated similarly.

In this work, spatial expression of *RcPT1* analysis showed that mRNA of this transporter could be detected in the roots, the stems and the leaves of 22 days *Ricinus* plants grown under Pi starvation (Figure 3.3.9). This result indicates that *RcPT1* may play a role as a Pi transporter protein in Pi translocation within the mature plants. In addition, the accumulation of dry matter in leaves and stems of *Ricinus* plants grown under Pi

starvation condition significantly decreased while the dry matter of roots increased 15% compared with that in Pi sufficient plants (Figure 3.2.3 A). It has been shown that length and density of root hairs were higher in plants grown under Pi starvation compared to Pi sufficient plants (Bates and Lynch, 1996). The increased volume of roots in Pi starved plants has the purpose to acquire more Pi in soil. High expression of *RcPT1* in plant's roots as a response to Pi starvation correlates with recent reports of increased Pi uptake rate of roots under Pi starvation. Rausch *et al*. (2001) had identified the localization of *StPT3* transporter from potato in the cells containing arbuscules within the roots of plants colonized by endomycorrhizal fungi. This indicates that *StPT3* transporter is involved in the primary uptake of Pi. Similarly, *LePT1* from tomato was expressed in all organs of plants, especially in roots (Daram *et al*., 1999). Further studies by using a specific LePT1 antibody have indicated that this transporter was associated with the plasma membrane prepared from the root of Pi starved plants. This result suggests that the levels of mRNA increase due to Pi deficiency are translated into LePT1 protein and this protein is targeted to the plasma membrane, thus the capacity of Pi uptake is increased (Daram *et al*., 1998; Muchhal and Raghothama, 1999). In addition, *LePT1* transcripts and protein showed similar expression patterns in the root of tomato plants grown in Pi starvation. The *MtPT1* transporter transcripts from *Medicago truncatula* were detected in the root epidermis, root hairs and root cap; Immunolocalization results indicated that this transporter has also been localized to the plasma membrane of these cells (Chiou *et al*., 2001).

The *in situ* hybridization results showed *RcPT1* transcripts in the root epidermis of *Ricinus* plants grown under Pi starvation (Figure 3.3.10). It seems that Pi starvation also leads to increase *RcPT1* transcripts in the root steles. In addition, immunological studies with the antibodies specific to *Ricinus* phosphate transporter revealed the accumulation of RcPT1 proteins in the root cap, epidermis and stele; similar results were also obtained in tomato *LePT1* and potato *StPT2* (Leggewie *et al*., 1997; Daram *et al*., 1998). These observations suggest that epidermally localized transporters play an important role in uptake of Pi from soil and the epidermal transfer cells may also be a factor in the active Pi uptake in the roots under Pi deficient conditions.

The nutrients were taken up from soil to plant via xylem, which locate in the central core (stele) of the roots. In order to reach the xylem nutrients must move through the epidermis-cortex, endodermis and stele cells. Furthermore, epidermis-cortex cells were

suggested to play a significant role in nutrient uptake while endodermis cells were involved in regulation of nutrient transport to the stele (Grunwald *et al*., 1979). The expression of *RcPT1* in the stele may be involved in increasing the capacity for Pi loading in the xylem, thus enhances Pi uptake from soil. It is reported that root Pi uptake capacity increase under Pi starvation is the result from an increase in V_{max} ,but not in the K_m (Clarkson and Scattergood, 1982; Colgliatti and Clarkson, 1983; Drew *et al*., 1984; Dunlop *et al*., 1997).

It is suggested that the boundary between the apoplasm and the symplasm of roots involved in nutrient uptake is the plasma membrane of root epidermal and cortical cells. The movement of nutrient ions from soil to the plant through the plasma membrane of the root epidermal and cortical cells is considered as a significant step in the nutrient transport, this membrane functions to both mediate and regulate nutrient uptake (Smith *et al*., 2003). The phosphate transporters are supposed to be involved in this transport of nutrients.

Although the nature of the transporter participating in Pi transport via xylem is still not clear, the existence of Pi transporters that are involved in xylem loading of Pi in roots were suggested in an *Arabidopsis* mutant (*pho1*) which lacks the ability to load Pi into the xylem, thus uploaded Pi from xylem into leaf tissues through the shoots is reduced in this mutant (Poirier *et al*., 1991). Liu *et al*. (1998a) reported that the expression of Pi transporter genes is not only induced by a rapid response to Pi starvation but also at an alteration of Pi status in the plants. The results from the divided root experiments provided the evidence of the significant role of internal Pi concentration in regulating the rates of Pi uptake by the roots and the distribution of Pi within the plant (Cogliatti *et al*., 1983; Drew and Saker, 1984). Moreover, in another split root experiments, in which tomato roots were halved and exposed separately, each part in either Pi deficiency or Pi sufficiency; the transcript levels of *LePT1* and *LePT2* were comparable to that found in plants grown in Pi sufficiency (Liu *et al*., 1998a). The total Pi concentration analysis in *Ricinus* plants grown in Pi sufficient conditions turned out to be distinctly higher than that of plants grown in Pi starvation, while the expression of *RcPT1* was only detected in Pi starved plants, suggesting the relationship between the relative expression of *RcPT1* and the concentrations of total Pi in *Ricinus* plants (Figure 3.2.3 B). These data reveal that the internal signal mechanisms leading to the expression of phosphate transporters are initiated in response to changes in internal concentration of Pi in plants.

The studies of the regulation as well as cellular distribution of members of the phosphate transporter family *Pht1* indicate that most of transporter genes belonging to this family are expressed in the roots under Pi starvation, suggesting their role in Pi uptake (Chiou *et al*., 2001; Karthikeyan *et al*., 2002; Mudge *et al*., 2002). However, the expression of some members of the *Pht1* family of phosphate transporters was also found in other plant tissues, suggesting that these transporters may be involved in the internal distribution of Pi within plants such as loading or unloading from the xylem or phloem, retranslocation of Pi from the old leaves or deposition into seed and storage tissue (Leggewie *et al*., 1997; Daram *et al*., 1998; Liu *et al*., 1998a; Rosewarne *et al*., 1999). This is consistent with obtained results from this work that even though belonging to *Pht1* family which includes transporter genes mainly expressed in the roots, transcripts of *RcPT1* were not only detected in the roots but also in the leaves and the stems of *Ricinus* plants grown in Pi starvation. However, *RcPT1* transcript accumulation was higher in the roots than in the leaves and the stem. Similarly, the expression of phosphate transporters was also detected in leaves, stem, tubers and flowers of potato, tomato and tobacco (Leggewie *et al*., 1997; Daram *et al*., 1998; Kai *et al*., 2002). In addition, *RcPT1* transporter has been localized to the upper epidermis, minor vein of leaves and stem vasculature including phloem cells. These findings suggest that *RcPT1* may function in loading Pi within the plant via phloem. Similar results have also been obtained from *LePT1* tomato (Liu *et al*., 1998a). Mimura *et al*. (1996) suggested that when transport of Pi from roots to the shoots was restricted under Pi starvation, stored phosphate is mobilized and retranslocated from the older leaves to the younger leaves and growing roots via phloem. Interestingly, about one-half of the Pi translocated from the shoots to the roots in the phloem is then transferred to the xylem and recycles back to the shoots (Jeschke *et al*., 1997).

Taken together, the obtained results show that *RcPT1* encodes a transporter with various Pi transport functions which is involved in the enhancement of Pi uptake under Pi starvation and the transport of Pi within plants, in addition to Pi acquisition by the roots.

4.2.3 Cloning and molecular characterization of *RcPS1* cDNA

Acid phosphatases are enzymes which catalyze the hydrolysis of the Pi reserves and exhibit a pH optimum below 6.0. APases are involved in the metabolic processes of

plant germination. Plants respond to Pi starvation by increasing the production of both extracellular and intracellular APase, while extracellular APase plays an important role in scavenging Pi from soil organics, intracellular APase is involved in remobilizing and regulating the supply of Pi from intracellular organic sources (Duff *et al*., 1994). Under Pi starvation, intracellular acid phosphatase and nuclease activities were increased corresponding to increased expression of their genes, suggesting the presence of a control at the level of transcription to Pi starvation. Luan (2003) suggested that the initiation of Pi specific signal cascades might be involved in response to Pi starvation due to expression of genes encoding phosphatase increase in Pi starvation. It may also play an important role in protein regulatory cascades. In addition, many changes in specific isoforms of APases in response to Pi starvation have been found (Trull and Deikman, 1998; Coello, 2002; Lim *et al*., 2003). Thus, APase activity has been used as a potential marker of the Pi status of plants (Ascencio, 1994).

Although the role of APases in plant metabolism and their response to Pi starvation have been intensively studied, only few APase genes and peptide sequences from plants have been identified so far (del Pozo *et al*., 1999; Wasaki *et al*., 1999b; Baldwin *et al*., 2001; Miller *et al*., 2001). In this study, based on amino acid sequences of known acid phosphatases, *RcPS1* was isolated from 6-day-old *Ricinus* seedlings grown under Pi starvation conditions by RT-PCR. The amino acid sequence of RcPS1 shows high similarity with recent identified phosphatases from *Lycopersicon esculentum* (Assesion number AAG40473); *Oryza sativa* (Assesion number NP_916687) and *Arabidopsis thaliana* (Assesion number AAM63155). The phylogenetic analysis between RcPS1 to phosphatase from other plants shows that RcPS1 shares 74% identity with LePS1 from tomato (Baldwin *et al*., 2001). *RcPS1* is 1116bp long and contains 747 bp open reading frame (ORF) encoding a peptide of 248 amino acids with a calculated molecular mass of 27,5 kD. Hydropathy plots of the deduced amino acid sequence indicated that RcPS1 is a soluble protein (Figure 3.4.3).

A homology search of amino acid sequences with RcPS1 and the other phosphatase clones revealed the highest degree of similarity (Figure 3.4.1). The comparison of RcPS1 with phosphatase from prokaryotes and eukaryotes suggested the presence of two highly conserved motifs, motif 1 “DFDXT” and motif 2 “GDGXXD”, which are typical for members of haloacid dehalogenase and DDDD superfamilies of enzymes catalyzing a diverse number of hydrolytic and phosphotransferase reactions (Thaller *et al*., 1998;

Aravind *et al*., 1998; Houston *et al*., 1999). The first of the two aspartates in the motif "DFDXT" is phosphorylated during phosphate transfer reaction; similar to dehalogenase, the aspartate residue of the phosphatases may bind the Pi group of their substrate. The amino acid sequence of motif 1 "DFDXT" is conserved in phosphomutases and phosphatases. While the motif 2 "GDGXXD" is mostly found in phosphatases rather than in phosphomutases (Collet *et al*., 1998). Moreover, homologs of these two conserved motifs sequence have been recognized in different organisms including vertebrates and plants (Baldwin *et al*., 2001). It is indicated that *RcPs1* may be involved in various functions in plants; however similar to *LePS1*, *RcPS1* belong to a novel family of phosphohydrolases with unknown enzymatic function (Baldwin *et al*., 2001). Therefore it is interesting to identify the native substrate(s) for hydrolysis by RcPS1 peptide and the biochemical pathway in which it is involved. The functional analysis of *RcPS1* was performed in *Escherichia coli* (*E.coli*) and the activity of induced APase in bacteria was assayed. The obtained results showed that the construct pET28-RcPS1 could express a recombinant protein with an expected mass as determined on SDS-PAGE, while *RcPS1* exhibited the capability in catalyzing the hydrolysis of *p*-nitrophenyl phosphate, a substrate of APase. Taken together, these results indicate that *RcPS1* can function in releasing Pi from organic sources, thus the expression of this gene could be involved in improving Pi acquisition or remobilization in plants.

4.2.4 Expression of acid phosphatase, *RcPS1* in *Ricinus* plants

The expression of *RcPS1* analysis showed that mRNA of this gene could be detected in the roots, the stem and the leaves of 22-day-old *Ricinus* plants grown under Pi starvation, no signal was obtained in plants grown in Pi sufficient conditions. This is in agreement with observed results from tomato *LePS2*, which was expressed in stems, petioles, leaves and roots (Baldwin *et al*., 2001). When tomato cells were transferred into Pi starvation medium, *LePS2* transcripts were induced rapidly; the rapid induction was obtained within three to twelve hours of transfer of cell cultures, indicating that *LePS1* belongs to the group of genes which are involved in the Pi starvation rescue process (Baldwin *et al*., 2001). In addition, the amount of excreted phosphatase in Pi-starved tomato cells was six times higher than that of Pi sufficient cells (Goldstein *et al*., 1988a). In all organisms, response of specific genes during Pi deficiency is considered as a

universal phenomenon. In plants, APases also increase activity under Pi deficiency. The secretion of acid phosphatase from *Arabidopsis* roots under Pi starvation was detected by staining the roots with the chromogenic substrate BCIP. The blue color reveals the presence of high APase activity in the roots (Trull *et al*., 1997; Trull and Deikman, 1998). In addition, similar activities have been obtained in the roots of rice, white lupine and tomato (Tomato and Sakai, 1991). These data revealed that *RcPS1* was induced in response to Pi starvation in *Ricinus* plants. Moreover, *RcPS1* was expressed in all organs of *Ricinus* plants, suggesting that *RcPS1* is involved in the internal mobilization of Pi. The expression pattern of *RcPS1* correlated with the analysis of the total Pi concentration in *Ricinus* plants grown either in Pi starvation or Pi sufficiency. In detail, the total Pi concentration in leaves, stems and roots of Pi-starved plants was much lower than that in Pi sufficiency; for example the measurement of the total Pi concentration in roots of Pi-starved plants was 3.2 times lower than that obtained from Pi sufficient plants (Figure 3.2.3 B). This implicated that the signaling system regulates the expression of *RcPS1* may depend on the internal Pi status.

The expression of *LePS2* was not detected in case of other deficient nutrients such as nitrogen, potassium and iron; however its expression was repressed when Pi was re-supplied to Pi-starved plant (Baldwin *et al*., 2001). Similarly, the Pi supplement to Pi-starved plants can reverse the induction of *AtACP5*, a type 5 APase gene, which was isolated and characterized based on purification of a 34-kD APase from *Arabidopsis* (del Pogo *et al*., 1999). The transcript of *AtACP5* was also observed in roots, shoots and senescent leaves of *Arabidopsis* grown under Pi starvation. The induction of *AtACP5* expression was also found in response to salt stress, oxidative stress and abscisic acid addition, whereas the expression of *AtACP5* was not induced by other phytohormones and other nutrient starvations, such as potassium or nitrogen. Transgenic *Arabidopsis* plants containing *AtACP5*: β-glucuronidase (GUS) translational fusion also showed high activity in response to the above mentioned stresses. These results suggest that *AtACP5* plays a role in internal remobilization of Pi in *Arabidopsis* plants (del Pozo *et al*., 1999). Another secreted APase gene, *AtsAPase*, was isolated from *Arabidopsis* (Haran *et al*., 2000). This gene was induced in roots under Pi starvation. *AtsAPase* promoter was fused to green fluorescent protein (GFP) as a reporter and the signal GFP was observed in root exudates of Pi starved plants (Haran *et al*., 2000).

In situ hybridization demonstrated the presence of *RcPS1* transcripts in the leaves, upper epidermis, mesophyll, and phloem cells, indicating that *RcPS1* might be involved in remobilization of endogenous Pi stored in leaves under Pi starvation (Figure 3.4.11). Furthermore, *RcPS1* transcript was also found in the shoot bundles; epidermis and stele of roots. The expression of *RcPS1* in these cell types may enhance Pi uptake efficiently in plants. These results implicate that *RcPS1* is induced in response to Pi starvation. In addition, *RcPS1* gene may play multiple roles in the plants including enhancing the availability and utilization of organic Pi in plants. This supports the report of Duff *et al*. (1994) that intracellular APases are involved in the routine utilization of stored Pi or Pi-containing compounds depending on their subcellular localization. It is possible that the expression of phosphate transporter *RcPT1* and *RcPS1* occurs in the same tissues such as leaves, stems or roots of Pi starved plants. This suggests that *RcPS1* may correlate with enhanced expression levels of *RcPT1* under Pi starvation. It has been suggested that Pi starvation induced genes are regulated by changing Pi levels in the plant tissues. Expression of three ribonuclease genes *RNS1*, *RNS2* and *RNS3* increased in Pi starved *Arabidopsis*, indicating that these genes may be involved in the internal remobilization of Pi (Hammond *et al*., 2004); ribonuclease may increase the Pi level in the plant by metabolizing ribonucleic acids. Moreover, the expression of ribonuclease genes is also supposed to be correlated with germination, senescence and differentiation of tracheary elements (Stenzel *et al*., 2003). The involvement of acid phosphatase in senescence has also been suggested by Duff *et al*. (1994).

Recently, three members of the *LePS2* gene family of acid phosphatases were isolated from cDNA library of tomato (*Lycopersicon esculentum*) cell cultures growing in media lacking Pi (Stenzel *et al*., 2003). The nucleotide sequence of these three genes, *LePS2A*, *LePS2B* and *LePS2C* shows similarity to each other except their C-terminal sequences and the 3´-untranslated regions. The expression of these three genes in Pi starved cell cultures and in Pi starved plant is similar to that of *LePS2* gene (Baldwin *et al*., 2001). However, during the first few days of germination of seedlings, the authors have found that these three genes are expressed at the same level in both conditions, with Pi or without Pi. Similarly, expression levels of *RcPS1* were also found to be similar under both conditions. Together, it is concluded that RcPS1 may represent an acid phosphatase in *Ricinus* plants.

4.3 The function of *RcPT1* and *RcPS1* in the germination of *Ricinus* seedlings

Germination of seeds is a complex including the combination of different biochemical processes, thus requiring the coordinated expression of various genes in the different tissues. Although, most of the genes expressed in seedlings are likely to differ from those in mature plants, a number of transcripts expressed at high levels in germinated seedlings are also found in developing seeds (Harada *et al*., 1988; Thomas, 1993; Bove *et al*., 2001). It has been suggested that in germinated seeds, storage products accumulated in reserve tissues are firstly mobilized in the periphery of endosperm and then relocated to support the growth of the seedlings (Mansfield and Briarty, 1996). During germination, Pi plays an important role in many reactions such as formation of various phosphate sugars and nucleotides for metabolism and energy producing processes and synthesis of nucleic acids. Therefore, a number of enzymes and genes involving in Pi are expected to be active exist in this period.

4.3.1 *RcPT1* gene functions as a phloem-specific phosphate transporter in germinated seedlings

In seeds, phytate (inositol hexaphosphate) plays a role as a central Pi source, which is deposited in the embryo and endosperms during seed development. During germination, phytic acid is degraded by phytases, subsequently releasing Pi which is remobilized to the growing part of seedlings (Lott *et al*., 1995). In germinated seedlings, there are no plasmodesmatal connections between the endosperms and maternal tissues. Thus Pi required for metabolic activity needs to be taken up by transporters in the plasma membranes of the peripheral endosperms (Mudge *et al*., 2002). On the other hand, it is supposed that Pi reserve in the apoplast of source organs, such as cotyledons or leaves, can be remobilized to sink organs such as hypocotyls and roots through the sieve element-companion cells during growth (Sondergaard *et al*., 2004); that is considered as a long-distance transport from source to sink organs, the phloem. However, not only mineral nutrients but also metabolites, such as sugars and amino acids, use phloem pathway. The *Ricinus communis* L. seedlings have been used as a model for analysis of short and long distance transport processes (Weig and Komor, 1996). Thus, the loading of Pi to the sieve element-companion cells is a significant physiological process for effective recycling of Pi nutrient. However, an active transport system in plasma

membranes is required for this loading process. In the studies of *Ricinus*, William and Gregory, (2004) reported that plasma membrane H^+-APase is highly expressed in the epidermis and the phloem of the cotyledons, suggesting the presence of nutrient uptake in these tissues during growth of seedlings.

In this study, the signal transcripts of *RcPT1* were observed in the endosperms, the cotyledons, the hypocotyl and the roots at 6-day-old *Ricinus* seedlings, indicating that *RcPT1* is involved in Pi remobilization within the seedlings. This is consistent with the recent results, in which the expression of the *Pht1;5* promoter was detected in the cotyledons and hypocotyls of 4-day-old *Arabidopsis* seedlings (Mudge *et al*., 2002). The low *RcPT1* transcript level was firstly detected in the hypocotyl and the roots of 3-day-old seedlings, and the endosperms of 4-day-old seedlings. Although, *RcPT1* was expressed in all organs of 6-day-old seedlings including the endosperm, the cotyledons, the hypocotyl and the roots, *RcPT1* transcript levels were different between these organs. The highest *RcPT1* transcript was detected in the endosperms and the roots of 6-day-old seedlings, while *RcPT1* transcript accumulated strongly in the cotyledons and the hypocotyl at day 7. This difference in expression may be due to the requirement of Pi between the organs of seedling. However, the transcript level of *RcPT1* was undetectable in the endosperms of 7-day-old seedlings. This may be due to the fact that almost all storage products were mobilized in this organ and transferred to the cotyledons. It is suggested that the required nutrients including Pi in sieve tube sap move from the cotyledons (as a source organ) to the growing seedling axis (as a sink organ) in growing *Ricinus* seedling (Komor *et al*., 1996). The expression pattern of *RcPT1* in different organs of the seedling indicates that this Pi transporter may plays a role in mobilization of Pi between source and sink organs of the *Ricinus* seedling.

Western blot analysis was carried out with antibody specific to RcPT1. It was shown that the RcPT1 protein levels correlated with the *RcPT1* transcript level in the seedlings during germination. Moreover, the regulation of *RcPT1* in this period is proposed to be independent on exogenous Pi supply based on the observation that similar transcript levels of *RcPT1* were obtained in both, Pi starvation and under Pi sufficiency. Recently, a similar result has been reported for the expression of the *LaPT1* gene from white lupin (Liu *et al*., 2005), which is expressed in the roots of seedlings grown in both Pi conditions, with or without Pi. However, the transcript of *LaPT1* was enhanced in presence of exogenous supplemented sugar.

The *in situ* hybridization and Immunolocalization results demonstrated the presence of *RcPT1* transcripts and protein in the adjacent area of endosperms to cotyledons; in the phloem and in the lower epidermis of cotyledons. In addition, *RcPT1* transcripts were also found in the phloem of hypocotyls and roots. These results indicated that *RcPT1* engaged in the transport of Pi from endosperms to cotyledons and the redistribution of Pi around seedlings via phloem during germination. It has been reported that besides the *Arabidopsis* mutant (*pho1*) involved in xylem loading (Poirier *et al*., 1991), the complexity of Pi transport in plants is represented by the existence of another specific phosphate transporter in phloem loading of Pi, the so-called *pho2* mutant of *Arabidopsis*. This mutant shows an inability of shoot cells in regulating internal Pi concentrations and thus leads to a high accumulation of Pi in leaves even under Pi starvation (Delhaize and Randall, 1995). This may be due to the alteration of function of Pi transporters in the shoots or to regulatory genes involved in Pi homeostasis in the shoots. This mutation indicates the significance of mechanism regulating intracellular phosphate concentrations in the plants.

It is supposed that phloem loading and unloading are important components of the internal movement of Pi in the plants. Based on the function of *RcPT1* involved in phloem loading, the present results can contribute to the knowledge on phosphate transporters in plants. However, a number of questions remain to answer which factor can activate the expression of *RcPT1* instead of Pi deficiency and whether germination conditions can regulate the gene expression. Moreover, the function of the *RcPT1* gene in phloem loading needs to be studied further.

4.3.2 Spatial and temporal expression of the acid phosphatase mRNA during germination and growth of seedlings

Germination of seeds is considered to be a multi-stage process, in which the coordination of numerous genes in different tissues is required. The expression of these genes is supposed to be coordinated and regulated both spatially and temporally by the different biochemical processes and functions of seed. Numerous enzyme activities were induced after imbibition of the seeds; these enzymes include proteinase, α-amylase, ribonuclease and phosphatase (Barker *et al*., 1974; Potokina *et al*., 2002). It is

suggested that after imbibition acid phosphatase activities increased in order to release the storage material in reserve tissues for the growth of seeding (Tamura *et al*., 1982; Haraguchi *et al*., 1990; Gibson and Ullah, 1998; Prazeres *et al*., 2004). It has been shown that APase and RNase activities of the tomato seedlings grown either in Pi sufficient or Pi deficient conditions similarly increased to the highest level at day four and then decreased to low level until day nine; after day ten the enzyme activities continuously decreased in Pi sufficient seedlings while increased again in Pi deficient seedlings. This second increase of the enzyme activities was probably caused by the low free Pi content in the Pi deficient seedlings (Bosse and Köck, 1998).

Although many kinetic studies have been reported on the purification of acid phosphatase from germinated seeds (Barker et al., 1974; Granjeiro *et al*., 1999; Bose and Taneja, 1998) very little information is available on the characterization and the function of phosphatase genes in this period.

To date, only two acid phosphatase genes *LePS2* from the tomato (Stenzel *et al*., 2003) and *LaSAP1* from white lupin (Liu *et al*., 2005) have been demonstrated to be involved in germination of seedlings. Moreover, *LePS2* and *LaSAP1* were also induced by Pi starvation (Baldwin *et al*., 2001; Miller *et al*., 2001). *LePS1* transcript accumulation was detected in the endosperms of 4-day-old seedlings grown in both Pi starvation and Pi sufficiency, whereas a low *LaSAP1* transcript accumulation was only detected in the roots of 5-day-old seedlings. However, the transcript level of *LaSAP1* gene increased significantly only in the presence of glucose, sucrose or fructose. It is suggested that exogenous sugar enhanced *LaSAP1* transcript accumulation (Liu *et al*., 2005). While the *RcPS1* transcript accumulation was found in different organs of 3-day-old *Ricinus* seedlings such as the endosperms, the cotyledons and the roots, no transcript was detected in the hypocotyl of seedlings during germination. The transcript level of *RcPS1* increased significantly in the endosperm and the roots during germination and reached the highest level at day 6. However, *RcPS1* transcript accumulation decreased in the roots of 7-day-old seedlings grown in Pi sufficiency, whereas it remained on a high level in seedlings grown in Pi starvation. The observation suggested that *RcPS1* gene might be expressed during mobilization of storage products in source organs such as the endosperm and the cotyledons; meanwhile this gene plays a role in the internal Pi remobilization during germination. The APase activities were increased in the beginning of germination; meanwhile Pi content was decreased, suggesting that in order to

maintain Pi level for all processes of germination, stored Pi and Pi-containing compounds as a substrate of APase was supposed to be hydrolyzed (Granjeiro *et al*., 1999).

Interestingly, the expression of *RcPS1* showed similar transcript levels between the same organs in germinated seeds grown either in Pi sufficiency or Pi starvation. This indicates that the expression of *RcPS1* was independent from the exogenous Pi supply. Similar results have also been found in *LePS2* from tomato seedlings (Stenzel *et al*., 2003) and *LaSAP1* from white lupin (Liu *et al*., 2005); different signal pathways influence the activities of APase genes indicating the distinct spatial and temporal expression of these genes (Stenzel *et al*., 2003). However, no signal was obtained in the hypocotyl of the *Ricinus* seedlings during this period indicating *RcPS1* gene may only function in a specific organ of seedlings. In contrast, *RcPT1* is strongly expressed in the hypocotyl due to its involvement in transport of Pi within seedlings during germination.

It has been reported that acid phosphatases are normally observed to be localized in the cytosol, vacuoles or cell walls (Duff *et al*., 1994). *In situ* hybridization studies showed the localization of *RcPS1* mRNA in the endosperm, the lower and upper epidermis of the cotyledons, the phloem of cotyledons and the phloem of roots from 6-day-old seedlings, indicating that *RcPS1* might be involved in mobilization of storage Pi during germination. Recently, the subcellular localization of ribonuclease LX (*RNase* LE) from tomato was detected in phloem tissues and the *RNase* LE transcript was detected in very early stages of phloem development in the cotyledons (Köck *et al*., 2004). In addition, subcellular localization and function studies revealed that *RNase* LE is involved in programmed cell death processes such as germination and senescence (Lehmann et al., 2001). It is implicated that *RcPS1* may be involved in these processes due to its expression in germinated seedlings. However, the question here is which factor regulates these genes during germination instead of Pi deficiency. Recent studies of the regulation of phosphate transporter, *LaPT1* and acid phosphates, *LaSAP1* by exogenous supplement sugars (Liu *et al*., 2005) should be considered for further study of *RcPT1* and *RcPS1* genes.

Taken together, it is concluded that *RcPT1* and *RcPS1* genes not only respond to Pi starvation but also function in mobilization of Pi during germination of *Ricinus* seedlings, thus they are involved in the regulation of seedling growth. In addition, these genes are

supposed to be involved in a number of physiological processes, thus play an important role in Pi and macromolecular recycling in plants. However, the signals responsible for *RcPT1* and *RcPS1* genes that are expressed in germinated *Ricinus* seedlings are different from those responsible for expression of these genes under Pi starvation.

4.4 Outlook

Pi is supposed to be transported to developing seeds via phloem after flowering, thus *RcPT1* may be involved in this Pi transport. Further studies in expression and localization of *RcPT1* in developing seeds need to be performed in order to get insight into the function of the corresponding protein during seed maturation. Immunolocalization studies need to be performed by using antibodies raised against RcPS1 in order to identify the localization of RcPS1 proteins at cellular levels. Subcellular localization will help to define the function of this enzyme in vivo. Because *RcPS1* belongs to a novel family of phosphohydrolases with unknown enzymatic function (Baldwin *et al*., 2001), the involvement of RcPS1 isoenzymes in the breakdown of endogenous substrates and biochemical pathways needs to be further studied. *RcPT1* and *RcPS1* cDNA fragments can be used as a probe to search related clones in cDNA library of Pi starved *Ricinus* plants. The promoter of Pi deficiency induced genes in higher plants shares common cis-acting elements for coordinated regulation of gene expression by Pi status (Ragothama, 1999; Rubio *et al*., 2001). Due to the fact that, *RcPT1* and *RcPS1* are both expressed under Pi starvation and during germination, an experiment for cloning the promoter region of these genes is required in order to get insight into the detailed mechanism and different regulation of these genes in response to Pi status in plants. In addition, analysis on transformation of *RcPT1* and *RcPS1* into *Ricinus* plants can provide more evidence for the contribution of these genes in the transport of Pi. Recently, Sujatha and Sailaja, (2005) were successful in the transformation of *Ricinus* via *Agrabacterium tumefaciens*, this might offer the possibility of co-suppressing or over-expressing these sequences in transgenic *Ricinus* to allow the evaluation of expected phenotypes under suitable conditions and enabling us to validate the identity and expression patterns of these genes. It is also interesting to identify the coordination of different genes which are expressed during germination.

5 Summary

Phosphate (Pi) is one of the essential macronutrients required for growth and development of plants. Pi plays an important role in various metabolic processes, such as photosynthesis, respiration, energy conservation, carbohydrate metabolism and signal transduction. Although various Pi starvation induced genes have been isolated from different plant species grown under conditions of Pi starvation, information about their functions during germination and growth of seedlings is still lacking. During germination Pi stored in the endosperm is mobilized and transported to growing organs of seedlings, thus a phosphate transporters and acid phosphatases are expected to be involved in these processes. The aim of this study was to clarify the translocation of Pi within the seedlings and to identify the involvement of phosphate transporters and acid phosphatases in the growth of seedlings.

Uptake into the phloem was analyzed by incubating the cotyledons in Pi. The movement of ^{32}P-labeled applied as an inorganic phosphate (Pi) was detected from the cotyledons to the hypocotyl, in particular to its apical hook near the cotyledons, suggesting that Pi moves from the *Ricinus communis* L. cotyledons through the hypocotyl via phloem and partially re-circulates in the xylem or leaks out through the roots. Therefore reducing the efflux could be as important for the plant as increasing the efficiency of the uptake mechanism. Following the Pi uptake into the roots, the translocation of ^{32}P-labeled Pi to the cotyledons through the hypocotyl via the xylem showed that a high amount of radiotracer accumulated in the cotyledons. The accumulated Pi in the cotyledons can be retranslocated to the roots via phloem.

This work describes the cloning of the phosphate transporter *RcPT1* and the acid phosphatase *RcPS1* genes by RT-PCR from *Ricinus* seedlings grown under Pi starvation conditions. Phosphate transporter *RcPT1* contains an open reading frame encoding a 530 amino acid polypeptide with a calculated molecular mass of 59 kD. The deduced amino acid sequence of *RcPT1* revealed 12 membrane-spanning domains with a central hydrophilic region and significant high similarity in other known high-affinity Pi transporters belonging to the *Pht1* family. The expression of *RcPT1* in the yeast high-affinity phosphate transporter mutant strain complemented the mutant and enhanced the cell growth significantly. Southern blot analysis showed that the *RcPT1* gene is present

as a single or low-copy gene in the *Ricinus* genome. The transcripts of *RcPT1* were expressed in the endosperm, cotyledons, hypocotyl and roots during germination. In detail *in situ* hybridization studies revealed *RcPT1* expression in the adjacent area of endosperm to cotyledon, in the phloem and in the lower epidermis of cotyledons; Immunolocalization analysis showed RcPT1 accumulation at the same sites as its mRNA. In addition, *RcPT1* transcripts were also found in the phloem of hypocotyl, and the epidermis and the steles of roots. These results implicated that *RcPT1* is involved in the movement of Pi from endosperms to cotyledons and the redistribution of Pi within seedlings via phloem during germination.

Acid phosphatase *RcPS1* shows a 747 bp open reading frame encoding a 248 amino acids polypeptide with a calculated molecular mass of 27,5 kD. Hydropathy plots of the deduced amino acid sequence indicated that RcPS1 is a soluble protein. The amino acid sequence of RcPS1 shares significant similarity with the acid phosphatase LePS2 from tomato and highly conserved motifs, which are typical for a member of haloacid dehalogenase and DDDD superfamilies of enzymes catalyzing a diverse number of hydrolytic and phosphotransferase reactions. The functional analysis after expression of *RcPS1* in *E.coli* showed significant acid phosphatase activity. The high transcript level of *RcPS1* in endosperms, cotyledons and roots at the first few days of germination suggested that this acid phosphatase gene might be expressed during mobilization of storage products. *RcPT1* and *RcPS1* mRNA are detectable in the seedlings grown under Pi starvation and Pi sufficient conditions, indicating that both genes were expressed independently from exogenous Pi supply during germination. Moreover, *RcPT1* and *RcPS1* were expressed in leaves, stems and roots of plants grown under Pi starvation; furthermore, *in situ* hybridization studies localized *RcPT1* and *RcPS1* mRNA in the epidermis and the stele of roots of Pi-starved plants, suggesting that these genes also play a role in response to Pi starvation. Thus, it is concluded that there are different signals regulating *RcPT1* and *RcPS1* expression in response to Pi starvation and during germination.

6 Zusammenfassung

Phosphat (Pi) zählt zu den wesentlichen Makronänrstoffen, die für die Pflanze zu deren Wachstum und Entwicklung benötigt werden. Pi spielt eine wichtige Rolle in verschiedenen metabolischen Prozessen, wie Fotosynthese, Atmung, Energieerhaltung, Kohlenhydratmetabolismus und Signaltrasduktion. Obgleich verschiedene durch Phosphat-Mangel induzierte Gene von verschiedenen Pflanzenarten isoliert wurden, ist noch immer wenig bekannt bezüglich ihrer Funktionen während der Keimung und des Wachstums der jungen Pflanzen. Während der Keimung wird im Endosperm gespeichertes Pi (source) mobilisiert und zu den wachsenden Organen der Keimlinge (sinks) transportiert. Dabei wird angenommen, dass Phosphattransporter- und Saure Phosphatase-Gene in diese Prozesse mit einbezogen sind. Die vorliegende Arbeit soll dazu beitragen, die Verteilung von Pi innerhalb der Keimlinge zu erklären und die Rolle der Phosphattransporter und Sauren Phosphatasen im Wachstum der Keimlinge zu identifizieren.

Die Richtung der Verteilung des als ^{32}P-radioaktiv applizierten anorganischen Phosphats (Pi) wurde von den Keimblättern zum Hypocotyl ermittelt, besonders zum Haken des Hypokotyls nahe den Keimblättern, was nahelegt, dass Pi von den *Ricinus* Keimblättern über das Phloem zum Hypokotyl transportiert wird, und entweder teilweise in das Xylem rezirkuliert, oder durch die Wurzeln austritt. Folglich könnte es für die Pflanze genauso wichtig sein den Verlust über die Wurzeln zu verringern, wie die Effizienz des Aufnahmemechanismus zu erhöhen. Aufnahmeversuche von ^{32}P-markiertem Phosphat über die Wurzel, ergaben dessen Transport in die Keimblätter durch das Hypokotyl über das Xylem, so dass sich eine große Menge von Radioaktivität in den Keimblättern angesammelt hat. Das angesammelte Pi in den Keimblättern kann dann übers Pholem in die Wurzeln zurückgeführt werden.

Diese Arbeit beschreibt die Klonierung der Phosphattransporter *RcPT1* und der Gene der Sauren Phosphatase *RcPS1* von *Ricinus* Keimlingen, welche in einem Phosphatmangel-Zustand angezogen wurden. Der Phosphattransporter *RcPT1* enthält ein offenes Leseraster, das ein Polypeptid von 530 Aminosäure mit einer errechneten molekularen Masse von 59 kD kodiert. Die abgeleitete Primärstruktur von *RcPT1* weist auf 12 membranüberspannende Regionen mit einem zentralen hydrophilen Bereich,

sowie auf eine signifikant hohe Ähnlichkeit zu anderen bekannten Hochaffinitäts-Pi Transportern, die zur Pht1-Familie gehören, hin. Die Expression von *RcPT1* in einer Hochaffinitäts-Phosphattransporter defizienten Hefe komplementierte die Mutation und beschleunigte das Zellwachstum signifikant. Eine Southern-Blot-Analyse zeigte, dass das *RcPT1*-Gen einzeln oder in wenigen Kopien im *Ricinus* Genom vorliegt. *RcPT1*-Transkripte wurden während der Keimung, im Endosperm, in den Keimblättern, im Hypocotyl und in den Wurzeln nachgewiesen. Des Weiteren wurde durch in-situ Hybridisierung die Expression von *RcPT1* im angrenzenden Bereich der Endosperme zu den Keimblättern; im Phloem und in der unteren Epidermis der Keimblätter aufgezeigt; die Immunolokalisations- Analyse ergab demnach eine Ansammlung von RcPT1 an den gleichen Orten wie dessen mRNA. Zusätzlich wurden Transkripte von *RcPT1* auch im Phloem von Hypokotylen und Wurzel, Epidermis und Stelen gefunden. Diese Resultate implizierten eine funktionale Rolle im Transport von Pi aus dem Endosperm in die Keimblätter, sowie in der weiteren Verteilung von Pi innerhalb der Keimlinge über das Phloem.

Saure Phosphatase *RcPS1* besitzt ein offenes Leseraster von 747 bp, das ein Polypeptid mit 248 Aminosäuren (27.5 kD) kodiert. ‚Hydropathy Plots' der abgeleiteten Aminosäurereihenfolge ergaben, dass RcPS1 ein lösliches Protein ist. Die Primärstruktur von RcPS1 weist deutliche Ähnlichkeit mit der Sauren Phosphatase LePS2 der Tomate auf und enthält konservierte Motive, die typisch sind für Mitglieder der *haloacid dehalogenase*-Familie und für sogenannte DDDD Superfamilien, die eine verschiedene Anzahl von Hydrolyse- und Phosphotransferase Reaktionen katalysieren. Die Funktionsanalyse von *RcPS1* nach Expression in *E.coli* ergab eine eindeutige saure Phosphatase-Aktivität. Die starke Transkription von *RcPS1* in den Endospermen, in den Keimblättern und in den Wurzeln an den ersten Tagen der Keimung lässt darauf schließen, dass das Gen dieser Sauren Phosphatase während der Mobilisierung der Speicherprodukte aktiv ist.

RcPT1 und *RcPS1* mRNA sind in Keimlingen nachweisbar, die sowohl unter Phosphatmangel als auch unter einem ausreichenden Phosphatangebot angezogen wurden. Diese Beobachtung zeigt, dass beide Gene unabhängig von der exogenen Phosphat-Versorgung während der Keimung exprimiert werden. Außerdem wurden *RcPT1* und *RcPS1* in Blättern, Stämmen und Wurzeln ausgewachsener Pflanzen transkribiert, die unter Phosphat-Mangel gewachsen sind; weiterhin konnten durch in-

situ Hybridisationstudien *RcPT1* und *RcPS1* mRNA in der Epidermis und in der Stele der Wurzeln an Phosphatmangel leidender Pflanzen lokalisiert werde, was nahelegt, dass diese Proteine auch in der Antwort auf Phosphatmangel eine Rolle spielen. Daraus wird gefolgert, dass unterschiedliche Signale zur Aktivierung der Expression von *RcPT1* und *RcPS1* existieren, welche in Form von Phosphat Mangel oder während der Keimung, auftreten.

7 References

Aravind, L., Galperin, M.Y. and Koonin, E.V. (1998) The catalytic domain of the p-type atpase has the haloacid dehalogenase fold. *Trends Biochem Sci*, **23**, 127-129

Ascencio, J. (1994) Acid-phosphatase as a diagnostic-tool. *Communications in Soil Science and Plant Analysis*, **25**, 1553-1564

Baek, S.H., Chung, I.M. and Yun, S.J. (2001) Molecular cloning and characterization of a tobacco leaf cDNA encoding a phosphate transporter. *Mol Cells*, **11**, 1-6

Baldwin, J.C., Karthikeyan, A.S. and Raghothama, K.G. (2001) LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. *Plant Physiol*, **125**, 728-37

Barker, G.R., Bray, C.M. and Walter, T.J. (1974) The development of ribonuclease and acid phosphatase during germination of Pisum arvense. *Biochem J*, **142**, 211-9

Bates, T.R., and Lynch, J.P. (1996) Stimulation of root hair elongation in *Arabidopsis thaliana* by low phosphorus availability. Plant Cell Environ. **19,** 529-538.

Bates, T.R. and Lynch, J.P. (1996) Stimulation of root hair elongation in arabidopsis thaliana by low phosphorus availability. *Plant Cell Environ.*, **19**, 529-538

Bauer-Ruckdeschel (2001) Der Keimblattapoplast von *Ricinus communis* L. Kinetische Wechselwirkungen zwischen der Substratkonzentration und dem Langstreckentransport.

Bieleski, R.L. (1973) Phosphate pools, phosphate transport, and phosphate availability. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **24**, 225-252

Biswas, T.K. and Cundiff, C. (1991) Multiple forms of acid-phosphatase in germinating-seeds of vigna-sinensis. *Phytochemistry*, **30**, 2119-2125

Bose, S.K. and Taneja, V. (1998) Induction of a germination specific, low molecular weight, acid phosphatase isozyme with specific phosphotyrosine phosphatase activity in lentil (lens esculenta) seeds. *Biochem Biophys Res Commun*, **250**, 629-634

Bosse, D. and Kock, M. (1998) Influence of phosphate starvation on phosphohydrolases during development of tomato seedlings. *Plant Cell Environ.*, **21**, 325-332

Bun-Ya, M., Nishimura, M., Harashima, S. and Oshima, Y. (1991) The PHO84 gene of Saccharomyces cerevisiae encodes an inorganic phosphate transporter. *Mol Cell Biol*, **11**, 3229-38

Bunya, M., Nishimura, M., Harashima, S. and Oshima, Y. (1991) The pho84 gene of saccharomyces-cerevisiae encodes an inorganic-phosphate transporter. *Mol Cell Biol*, **11**, 3229-3238

Chiou, T.J., Liu, H. and Harrison, M.J. (2001) The spatial expression patterns of a phosphate transporter (MtPT1) from Medicago truncatula indicate a role in phosphate transport at the root/soil interface. *Plant J*, **25**, 281-93

Chung, C.C., Hwang, S.P. and Chang, J. (2003) Identification of a high-affinity phosphate transporter gene in a prasinophyte alga, Tetraselmis chui, and its expression under nutrient limitation. *Appl Environ Microbiol*, **69**, 754-9

Clarkson, D.T. and Hawkesford, M.J. (1993) Molecular biological approaches to plant nutrition. *Plant and Soil*, **156**, 21-31

Clarkson, D.T. and Scattergood, C.B. (1982) Growth and phosphate-transport in barley and tomato plants during the development of, and recovery from, phosphate-stress. *J. Exp. Bot.*, **33**, 865-875

Coello, P. (2002) Purification and characterization of secreted acid phosphatase in phosphorus-deficient arabidopsis thaliana. *Physiologia Plantarum*, **116**, 293-298

Cogliatti, D.H. and Clarkson, D.T. (1983) Physiological-changes in, and phosphate-uptake by potato plants during development of, and recovery from phosphate deficiency. *Physiologia Plantarum*, **58**, 287-294

Cogliatti, D.H. and Santamaria, G.E. (1990) Influx and efflux of phosphorus in roots of wheat plants in non-growth-limiting concentrations of phosphorus. *J. Exp. Bot.*, **41**, 601-607

Collet, J.F., Stroobant, V., Pirard, M., Delpierre, G. and Van Schaftingen, E. (1998) A new class of phosphotransferases phosphorylated on an aspartate residue in an amino-terminal dxdx(t/v) motif. *J. Biol. Chem.*, **273**, 14107-14112

Daram, P., Brunner, S., Persson, B.L., Amrhein, N. and Bucher, M. (1998) Functional analysis and cell-specific expression of a phosphate transporter from tomato. *Planta*, **206**, 225-33

Daram, P., Brunner, S., Rausch, C., Steiner, C., Amrhein, N. and Bucher, M. (1999) Pht2;1 encodes a low-affinity phosphate transporter from Arabidopsis. *Plant Cell*, **11**, 2153-66

del Pozo JC, Allona I, Rubio V, Leyva A, de la Pena A, Aragoncillo C, Paz-Ares J. , Muchhal, U.S., Pardo, J.M. and Raghothama, K.G. (1999) A type 5 acid phosphatase gene

from Arabidopsis thaliana is induced by phosphate starvation and by some other types of phosphate mobilising/oxidative stress conditions. *Plant J.*, **19 (5)**, 579-89

Delhaize, E. and Randall, P.J. (1995) Characterization of a phosphate-accumulator mutant of arabidopsis-thaliana. *Plant Physiol.*, **107**, 207-213

Dietz, K.J., Heber, U. and Mimura, T. (1998) Modulation of the vacuolar H+-ATPase by adenylates as basis for the transient CO2-dependent acidification of the leaf vacuole upon illumination. *Biochim Biophys Acta*, **1373**, 87-92

Drew, M.C. and Saker, L.R. (1984) Uptake and long-distance transport of phosphate, potassium and chloride in relation to internal ion concentrations in barley - evidence of non-allosteric regulation. *Planta*, **160**, 500-507

Duff, S.M.G., Moorhead, G.B.G., Lefebvre, D.D. and Plaxton, W.C. (1989) Phosphate starvation inducible bypasses of adenylate and phosphate dependent glycolytic-enzymes in brassica-nigra suspension cells. *Plant Physiol.*, **90**, 1275-1278

Duff, S.M.G., Plaxton, W.C. and Lefebvre, D.D. (1991) Phosphate-starvation response in plant-cells - denovo synthesis and degradation of acid-phosphatases. *Proc. Natl. Acad. Sci. USA*, **88**, 9538-9542

Duff, S.M.G., Sarath, G. and Plaxton, W.C. (1994) The role of acid-phosphatases in plant phosphorus-metabolism. *Physiologia Plantarum*, **90**, 791-800

Dunlop, J., Phung, H.T., Meeking, R. and White, D.W.R. (1997) The kinetics associated with phosphate absorption by arabidopsis and its regulation by phosphorus status. *Australian Journal of Plant Physiology*, **24**, 623-629

Elliott, G.C., Lynch, J. and Lauchli, A. (1984) Influx and efflux of p in roots of intact maize plants - double-labeling with p-32 and p-33. *Plant Physiol.*, **76**, 336-341

Fohse, D., Claassen, N. and Jungk, A. (1988) Phosphorus efficiency of plants .1. External and internal p requirement and p uptake efficiency of different plant-species. *Plant and Soil*, **110**, 101-109

Furihata, T., Suzuki, M. and Sakurai, H. (1992) Kinetic characterization of 2 phosphate-uptake systems with different affinities in suspension-cultured catharanthus-roseus protoplasts. *Plant Cell. Physiol.*, **33**, 1151-1157

Gahoonia, T.S. and Nielsen, N.E. (1998) Direct evidence on participation of root hairs in phosphorus (p-32) uptake from soil. *Plant and Soil*, **198**, 147-152

Gardner, W.K., Parbery, D.G. and Barber, D.A. (1982) The acquisition of phosphorus by lupinus-albus l .1. Some characteristics of the soil root interface. *Plant and Soil*, **68**, 19-32

Gibson, D.M. and Ullah, A.H.J. (1988) Purification and characterization of phytase from cotyledons of germinating soybean seeds. *Arch Biochem Biophys*, **260**, 503-513

Gilbert, G.A., Knight, J.D., Vance, C.P. and Allan, D.L. (1999) Acid phosphatase activity in phosphorus-deficient white lupin roots . *Plant Cell Environ.*, **22**, 801-810

Goldstein, A.H., Danon, A., Baertlein, D.A. and Mcdaniel, R.G. (1988) Phosphate starvation inducible metabolism in lycopersicon-esculentum .2. Characterization of the phosphate starvation inducible-excreted acid-phosphatase. *Plant Physiol.*, **87**, 716-720

Goldstein, A.H., Mayfield, S.P., Danon, A. and Tibbot, B.K. (1989) Phosphate starvation inducible metabolism in lycopersicon-esculentum .3. Changes in protein secretion under nutrient stress. *Plant Physiol.*, **91**, 175-182

Granjeiro, P.A., Ferreira, C.V., Granjeiro, J.M., Taga, E.M. and Aoyama, H. (1999) Purification and kinetic proper ties of a castor bean seed acid phosphatase containing sulfhydryl groups. *Physiologia Plantarum*, **107**, 151-158

Green, P.J. (1994) The ribonucleases of higher-plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **45**, 421-445

Greenwood, J.S. and Bewley, J.D. (1984) Subcellular-distribution of phytin in the endosperm of developing castor bean - a possibility for its synthesis in the cytoplasm prior to deposition within protein bodies. *Planta*, **160**, 113-120

Hammond, J.P., Broadley, M.R. and White, P.J. (2004) Genetic responses to phosphorus deficiency. *Ann Bot (Lond)*, **94**, 323-32

Harada, J.J., Baden, C.S. and Comai, L. (1988) Spatially regulated genes expressed during seed-germination and postgerminative development are activated during embryogeny. *Molecular & General Genetics*, **212**, 466-473

Haraguchi, H., Yamauchi, D. and Minamikawa, T. (1990) Multiple forms of acid-phosphatase in cotyledons of vigna-mungo seedlings - immunological detection and quantitation. *Plant Cell. Physiol.*, **31**, 917-923

Haran, S., Logendra, S., Seskar, M., Bratanova, M. and Raskin, I. (2000) Characterization of Arabidopsis acid phosphatase promoter and regulation of acid phosphatase expression. *Plant Physiol*, **124**, 615-26

Harris, R.M., Webb, D.C., Howitt, S.M. and Cox, G.B. (2001) Characterization of pita and pitb from escherichia coli. *J Bacteriol*, **183**, 5008-5014

Harrison, M.J. (1999) MOLECULAR AND CELLULAR ASPECTS OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS. *Annu Rev Plant Physiol Plant Mol Biol*, **50** , 361-389

Hegeman, C.E. and Grabau, E.A. (2001) A novel phytase with sequence similarity to purple acid phosphatases is expressed in cotyledons of germinating soybean seedlings. *Plant Physiol.*, **126**, 1598-1608

Holford, I.C.R. (1997) Soil phosphorus: its measurement, and its uptake by plants. *Australian Journal of Soil Research*, **35**, 227-239

Ibrahim, H., Pertl, H., Pittertschatscher, K., Fadl-Allah, E., el-Shahed, A., Bentrup, F.W. and Obermeyer, G. (2002) Release of an acid phosphatase activity during lily pollen tube growth involves components of the secretory pathway. *Protoplasma*, **219**, 176-83

Jeschke, W.D., Kirkby, E.A., Peuke, A.D., Pate, J.S. and Hartung, W. (1997) Effects of p deficiency on assimilation and transport of nitrate and phosphate in intact plants of castor bean (ricinus communis l). *J. Exp. Bot.*, **48**, 75-91

Johnson, J.F., Vance, C.P. and Allan, D.L. (1996) Phosphorus deficiency in lupinus albus - altered lateral root development and enhanced expression of phosphoenolpyruvate carboxylase. *Plant Physiol.*, **112**, 31-41

Kai, M., Masuda, Y., Kikuchi, Y., Osaki, M. and Tadano, T. (1997) Isolation and characterization of a cdna from catharanthus roseus which is highly homologous with phosphate transporter. *Soil Science and Plant Nutrition*, **43**, 227-235

Kai, M., Takazumi, K., Adachi, H., Wasaki, J., Shinano, T. and Osaki, M. (2002) Cloning and characterization of four phosphate transporter cdnas in tobacco. *Plant Sci.*, **163**, 837-846

Karthikeyan, A.S., Varadarajan, D.K., Mukatira, U.T., D'Urzo, M.P., Damsz, B. and Raghothama, K.G. (2002) Regulated expression of Arabidopsis phosphate transporters. *Plant Physiol*, **130**, 221-33

Keerthisinghe, G., Hocking, P.J., Ryan, P.R. and Delhaize, E. (1998) Effect of phosphorus supply on the formation and function of proteoid roots of white lupin (lupinus albus l.). *Plant Cell Environ.*, **21**, 467-478

Kock, M., Gross, N., Stenzel, I. and Hause, G. (2004) Phloem-specific expression of the wound-inducible ribonuclease le from tomato (lycopersicon esculentum cv. Lukullus). *Planta*, **219**, 233-242

Komor, E., Orlich, G., Weig, A. and Kockenberger, W. (1996) Phloem loading - not metaphysical, only complex: towards a unified model of phloem loading. *J. Exp. Bot.*, **47**, 1155-1164

Kyte, J. & Doolittle, R. F. (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol ,**157**, 105-132

Lee, R.B. (1988) Phosphate influx and extracellular phosphatase-activity in barley roots and rose cells. *New Phytologist*, **109**, 141-148

Lefebvre, D.D., Duff, S.M.G., Fife, C.A., Julieninalsingh, C. and Plaxton, W.C. (1990) Response to phosphate deprivation in brassica-nigra suspension cells - enhancement of intracellular, cell-surface, and secreted phosphatase-activities compared to increases in pi-absorption rate. *Plant Physiol.*, **93**, 504-511

Leggewie, G., Willmitzer, L. and Riesmeier, J.W. (1997) Two cdnas from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. *Plant Cell*, **9**, 381-392

Lehmann, K., Hause, B., Altmann, D. and Kock, M. (2001) Tomato ribonuclease lx with the functional endoplasmic reticulum retention motif hdef is expressed during programmed cell death processes, including xylem differentiation, germination, and senescence. *Plant Physiol.*, **127**, 436-449

Lenburg, M.E. and O'Shea, E.K. (2001) Genetic evidence for a morphogenetic function of the Saccharomyces cerevisiae Pho85 cyclin-dependent kinase. *Genetics*, **157**, 39-51

Liu, C., Muchhal, U.S., Uthappa, M., Kononowicz, A.K. and Raghothama, K.G. (1998a) Tomato phosphate transporter genes are differentially regulated in plant tissues by phosphorus. *Plant Physiol*, **116**, 91-9

Liu, H., Trieu, A.T., Blaylock, L.A. and Harrison, M.J. (1998b) Cloning and characterization of two phosphate transporters from Medicago truncatula roots: regulation in response to phosphate and to colonization by arbuscular mycorrhizal (AM) fungi. *Mol Plant Microbe Interact*, **11**, 14-22

Liu, J., Samac, D.A., Bucciarelli, B., Allan, D.L. and Vance, C.P. (2005) Signaling of phosphorus deficiency-induced gene expression in white lupin requires sugar and phloem transport. *Plant J*, **41**, 257-68

Liu, J.Q., Uhde-Stone, C., Li, A., Vance, C. and Allan, D. (2001) A phosphate transporter with enhanced expression in proteoid roots of white lupin (lupinus albus l.). *Plant and Soil*, **237**, 257-266

Lott, J.N.A., Greenwood, J.S. and Vollmer, C.M. (1982) Mineral reserves in castor beans - the dry seed. *Plant Physiol.*, **69**, 829-833

Lott, J.N.A., West, M.M., Clark, B. and Beecroft, P. (1995) Changes in the composition of globoids in castor bean cotyledons and endosperm during early seedling growth with and without complete mineral nutrients. *Seed Science Research*, **5**, 121-125

Luan, S. (2003) Protein phosphatases in plants. *Annu Rev Plant Biol*, **54**, 63-92

Lynch, J. (1995) Root architecture and plant productivity. *Plant Physiol.*, **109**, 7-13

Mansfield, S.G. and Briarty, L.G. (1996) The dynamics of seedling and cotyledon cell development in arabidopsis thaliana during reserve mobilization. *International Journal of Plant Sciences*, **157**, 280-295

Maruyama, K. and Sugano, S. (1994) Oligo-capping - a simple method to replace the cap structure of eukaryotic messenger-rnas with oligoribonucleotides. *Gene*, **138**, 171-174

Maugenest, S., Martinez, I. and Lescure, A.M. (1997) Cloning and characterization of a cdna encoding a maize seedling phytase. *Biochem J*, **322**, 511-517

Miller, S.S., Liu, J.Q., Allan, D.L., Menzhuber, C.J., Fedorova, M. and Vance, C.P. (2001) Molecular control of acid phosphatase secretion into the rhizosphere of proteoid roots from phosphorus-stressed white lupin. *Plant Physiol.*, **127**, 594-606

Mimura, T. (1999) Regulation of Phosphate Transport and Homeostasis in Plant Cells. International Review of Cytology-a Survey of Cell Biology. Notes: Full Source Title: International Review of Cytology - a Survey of Cell Biology, **191**, 149-200.

Mimura, T. (1995) Homeostasis and transport of inorganic-phosphate in plants. *Plant Cell. Physiol.*, **36**, 1-7

Mimura, T., Sakano, K. and Shimmen, T. (1996) Studies on the distribution, re-translocation and homeostasis of inorganic phosphate in barley leaves. *Plant Cell Environ.*, **19**, 311-320

Mimura, T., Yin, Z.H., Wirth, E. and Dietz, K.J. (1992) Phosphate-transport and apoplastic phosphate homeostasis in barley leaves. *Plant Cell. Physiol.*, **33**, 563-568

Mitsukawa, N., Okumura, S., Shirano, Y., Sato, S., Kato, T., Harashima, S. and Shibata, D. (1997) Overexpression of an Arabidopsis thaliana high-affinity phosphate transporter gene in tobacco cultured cells enhances cell growth under phosphate-limited conditions. *Proc Natl Acad Sci U S A*, **94**, 7098-102

Muchhal, U.S., Pardo, J.M. and Raghothama, K.G. (1996) Phosphate transporters from the higher plant Arabidopsis thaliana. *Proc Natl Acad Sci U S A*, **93**, 10519-23

Muchhal, U.S. and Raghothama, K.G. (1999) Transcriptional regulation of plant phosphate transporters. *Proc Natl Acad Sci U S A*, **96**, 5868-72

Mudge, S.R., Rae, A.L., Diatloff, E. and Smith, F.W. (2002) Expression analysis suggests novel roles for members of the pht1 family of phosphate transporters in arabidopsis. *Plant J.*, **31**, 341-353

Ogawa, N., Derisi, J. and Brown, P.O. (2000) New components of a system for phosphate accumulation and polyphosphate metabolism in saccharomyces cerevisiae revealed by genomic expression analysis. *Mol Biol Cell*, **11**, 4309-4321

Olah, Z., Lehel, C., Anderson, W.B., Eiden, M.V. and Wilson, C.A. (1994) The cellular receptor for gibbon ape leukemia-virus is a novel high-affinity sodium-dependent phosphate transporter. *J. Biol. Chem.*, **269**, 25426-25431

Oneill, E.M., Kaffman, A., Jolly, E.R. and Oshea, E.K. (1996) Regulation of pho4 nuclear localization by the pho80-pho85 cyclin-cdk complex. *Science*, **271**, 209-212

Organ, M.G., Greenwood, J.S. and Bewley, J.D. (1988) Phytin is synthesized in the cotyledons of germinated castor-bean seeds in response to exogenously supplied phosphate. *Planta*, **174**, 513-517

Oshima, Y., Ogawa, N. and Harashima, S. (1996) Regulation of phosphatase synthesis in saccharomyces cerevisiae - a review. *Gene*, **179**, 171-177

Pao, S.S., Paulsen, I.T. and Saier, M.H. (1998) Major facilitator superfamily. *Microbiol Mol Biol Rev*, **62**, 1-+

Paszkowski, U., Kroken, S., Roux, C. and Briggs, S.P. (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proc Natl Acad Sci U S A*, **99**, 13324-9

Persson, B.L., Berhe, A., Fristedt, U., Martinez, P., Pattison, J., Petersson, J. and Weinander, R. (1998) Phosphate permeases of saccharomyces cerevisiae. *Biochimica Et Biophysica Acta-Bioenergetics*, **1365**, 23-30

Poirier, Y., Thoma, S., Somerville, C. and Schiefelbein, J. (1991) Mutant of Arabidopsis Deficient in Xylem Loading of Phosphate. *Plant Physiol*, **97**, 1087-1093

Potokina, E., Sreenivasulu, N., Altschmied, L., Michalek, W. and Graner, A. (2002) Differential gene expression during seed germination in barley (Hordeum vulgare L.). *Funct Integr Genomics*, **2**, 28-39

Raghothama, K.G. (1999) PHOSPHATE ACQUISITION. *Annu Rev Plant Physiol Plant Mol Biol*, **50**, 665-693

Rausch, C., Daram, P., Brunner, S., Jansa, J., Laloi, M., Leggewie, G., Amrhein, N. and Bucher, M. (2001) A phosphate transporter expressed in arbuscule-containing cells in potato. *Nature*, **414**, 462-70

Reid, R.J., Mimura, T., Ohsumi, Y., Walker, N.A. and Smith, F.A. (2000) Phosphate uptake in chara: membrane transport via na/pi cotransport. *Plant Cell Environ.*, **23**, 223-228

Richardson AE (1994) Soil microorganisms and phosphorus availability. *Soil Biota*, **50** , 62-66

Rosewarne, G.M., Barker, S.J., Smith, S.E., Smith, F.A. and Schachtman, D.P. (1999) A lycopersicon esculentum phosphate transporter (lept1) involved in phosphorus uptake from a vesicular-arbuscular mycorrhizal fungus. *New Phytologist*, **144**, 507-516

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A. and Paz-Ares, J. (2001) A conserved myb transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes & Development*, **15**, 2122-2133

Sakano, K. (1990) Proton phosphate stoichiometry in uptake of inorganic-phosphate by cultured-cells of catharanthus-roseus (l) g-don. *Plant Physiol.*, **93**, 479-483

Schachtman, D.P., Reid, R.J. and Ayling, S.M. (1998) Phosphorus uptake by plants: from soil to cell. *Plant Physiol.*, **116**, 447-453

Schmidt, M.E., Heim, S., Wylegalla, C., Helmbrecht, C. and Wagner, K.G. (1992) Characterization of phosphate-uptake by suspension cultured catharanthus-roseus cells. *Journal of Plant Physiology*, **140**, 179-184

Schobert, C. and Komor, E. (1992) Transport of nitrate and ammonium into the phloem and the xylem of ricinus-communis seedlings. *Journal of Plant Physiology*, **140**, 306-309

Shaefer, B. (1995) Revolution of rapid amplification of cDNAends: new strategies for polymerase chain rection cloning of full lenght cDNA ends. *Analytical Biochem*, **227**, 255-273

Smith, F.W., Ealing, P.M., Dong, B. and Delhaize, E. (1997) The cloning of two Arabidopsis genes belonging to a phosphate transporter family. *Plant J*, **11**, 83-92

Smith, F.W., Mudge, S.R., Rae, A.L. and Glassop, D. (2003) Phosphate transport in plants. *Plant and Soil*, **248**, 71-83

Smith, F.W., Rae, A.L. and Hawkesford, M.J. (2000) Molecular mechanisms of phosphate and sulphate transport in plants. *Biochimica Et Biophysica Acta-Biomembranes*, **1465**, 236-245

Smith, S.E. and Barker, S.J. (2002) Plant phosphate transporter genes help harness the nutritional benefits of arbuscular mycorrhizal symbiosis. *Trends Plant Sci*, **7**, 189-90

Snapp, S.S. and Lynch, J.P. (1996) Phosphorus distribution and remobilization in bean plants as influenced by phosphorus nutrition. *Crop Science*, **36**, 929-935

Sondergaard, T.E., Schulz, A. and Palmgren, M.G. (2004) Energization of transport processes in plants. roles of the plasma membrane H+-ATPase. *Plant Physiol*, **136**, 2475-82

Stenzel, I., Ziethe, K., Schurath, J., Hertel, S.C., Bosse, D. and Kock, M. (2003) Differential expression of the LePS2 phosphatase gene family in response to phosphate availability, pathogen infection and during development. *Physiol Plant*, **118**, 138-146

Sujatha, M., Sailaja M., (2005) Stable genetic transformation of castor (Ricinus communis L.) via Agrobacterium tumefaciens-mediated gene transfer using embryo axes from mature seeds. Plant Cell Rep. **23,** 803–810

Tadano, T. and Sakai, H. (1991) Secretion of acid-phosphatase by the roots of several crop species under phosphorus-deficient conditions. *Soil Science and Plant Nutrition*, **37**, 129-140

Thaller, M.C., Schippa, S. and Rossolini, G.M. (1998) Conserved sequence motifs among bacterial, eukaryotic, and archaeal phosphatases that define a new phosphohydrolase superfamily. *Protein Sci.*, **7**, 1647-1652

Thomas, T.L. (1993) Gene-expression during plant embryogenesis and germination - an overview. *Plant Cell*, **5**, 1401-1410

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res, **25**, 4876-4882

Trull, M.C. and Deikman, J. (1998) An Arabidopsis mutant missing one acid phosphatase isoform. *Planta*, **206**, 544-50

Trull, M.C., Knupp, B.J. and Deikman, J. (1997) Characterization of phosphatase-underproducing (pup) mutants of arabidopsis. *Plant Physiol.*, **114**, 509

Turner, W.L. and Plaxton, W.C. (2001) Purification and characterization of banana fruit acid phosphatase. *Planta*, **214**, 243-9

Ullrich, C.I. and Novacky, A.J. (1990) Extracellular and intracellular ph and membrane-potential changes induced by k+, cl-, h2po4-, and no3- uptake and fusicoccin in root hairs of limnobium-stoloniferum. *Plant Physiol.*, **94**, 1561-1567

Ullricheberius, C.I., Novacky, A., Fischer, E. and Luttge, U. (1981) Relationship between energy-dependent phosphate-uptake and the electrical membrane-potential in lemna-gibba g1. *Plant Physiol.*, **67**, 797-801

Ullricheberius, C.I., Novacky, A. and Vanbel, A.J.E. (1984) Phosphate-uptake in lemna-gibba-g1 - energetics and kinetics. *Planta*, **161**, 46-52

Wasaki, J., Omura, M., Osaki, M., Ito, H., Matsui, H., Shinano, T. and Tadano, T. (1999) Structure of a cdna for an acid phosphatase from phosphate-deficient lupin (lupinus albus l.) Roots. *Soil Science and Plant Nutrition*, **45**, 439-449

Weig, A. and Komor, E. (1996) An active sucrose carrier (scr1) that is predominantly expressed in the seedling of ricinus communis l. *Journal of Plant Physiology*, **147**, 685-690

Williams, L.E. and Gregory, A. (2004) Changes in the expression pattern of the plasma membrane h+-atpase in developing ricinus communis cotyledons undergoing the sink/source transition. *Planta*, **218**, 562-568

Zakhleniuk, O.V., Raines, C.A. and Lloyd, J.C. (2001) pho3: a phosphorus-deficient mutant of Arabidopsis thaliana (L.) Heynh. *Planta*, **212**, 529-34

Acknowledgment

First of all, I would like to express my warmest thanks to my supervisor Prof. Dr. Ewald Komor for giving me the possibility to work in his laboratory, for his advice, support and direction throughout my research.

I am very grateful to Dr. Alfons Weig for his brilliant advice and discussions in the beginning of this project.

I would like to thank PD Dr. Christiane Reinbothe, Dr. Heike Bauer-Ruckdeschel, Dr. Tribikram Bhattarai for the useful discussions and for the critical reading of my dissertation.

I am deeply thankful to Dr Sebastian Fettig, Dr. Katja Hartig, Dr. Daniel Eisenbarth, Mr. Frank Buhr, Mr. Armin Springer, Mr. Ulrich Sentner and all my colleagues from the Institute of Plant physiology, University of Bayreuth for their numerous advices, for the friendly cooperation and for making my stay in Bayreuth unforgettable. I am deeply thankful to Mrs. Pia Schuster for her friendly and excellent technical assistance in the laboratory. Special thanks to Dr. Katja Hüve, for her help to perform the translocation of Pi experiments.

I also would like to thank Dr. Georg Leggewie in Max-Planck-Institute for Molecular Plant physiology, Golm, for providing yeast phosphate uptake mutant strains MB 192.

I wish to thank all my friends and my country-mates in Bayreuth for their support and inestimable friendship.

Finally, I thank my parents, brother and sister for their infinite support, encouragement and love during my stay in Germany.

Erklärung

Hiermit erkläre ich, dass ich die Arbeit selständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich nicht anderweitig mit oder ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen.

Bayreuth, den 15.11.2007

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