Chapter 4
Molecular Approaches to the Study of Biological Phosphorus Cycling

Jun Wasaki and Hayato Maruyama

4.1 Introduction

The organisms involved in phosphorus (P) cycling in soils are highly varied, and microorganisms probably play the most important role. However, more than 99% of soil microorganisms have not been cultured successfully (Torsvik and Øvreås 2002). Therefore, culture-independent methods are required to study the function and ecology of microbes involved in P cycling in soils. Molecular approaches for such culture-independent methods have been developed in the recent past.

Exudates from plant roots have significant effects on soil microbial ecology and P dynamics in the rhizosphere. Therefore, it is important to understand plant–microbe interactions, e.g., symbiosis with mycorrhizal fungi. Plant function and plant–microbe interactions can also be analyzed using molecular approaches.

In this chapter, the applications of molecular tools to study the role of plants and rhizosphere microorganisms in P cycling are discussed. Table 4.1 summarizes the molecular and biochemical tools introduced in this chapter. Since there are some advantages and disadvantages for each method, researchers should select the most appropriate tool(s) for their purpose.

4.2 DNA Extraction

4.2.1 Soil Samples

The starting point for all molecular approaches is the extraction of environmental DNA from the soil. We frequently face difficulties extracting DNA from soils, because soil properties vary strongly in terms of pH and content of mineral and

J. Wasaki (✉) and H. Maruyama
Graduate School of Biosphere Science, Hiroshima University, Kagamiyama 1-7-1, Higashi-Hiroshima 739-8521, Japan
e-mail: junw@hiroshima-u.ac.jp

E.K. Bünemann et al. (eds.), *Phosphorus in Action*, Soil Biology 26,
DOI 10.1007/978-3-642-15271-9_4,

Table 4.1 Summary of molecular and biochemical tools introduced in this chapter

Molecular tools	Dependency on PCR	Advantage	Disadvantage
DNA-based techniques	–	Ease of use for many approaches	Only the abundant organisms can be detected
RNA-based techniques	–	Active organisms can be detected	Application is difficult because of instability of RNA
Clone library	Yes	Information of DNA sequence for each clone becomes available	Relatively expensive
RFLP (restriction fragment length polymorphism)	Yes	Differences among samples can be detected at genus or higher levels	Sequence information is frequently too short for classification
DGGE (denaturant gradient gel electrophoresis)	Yes	Differences among samples can be detected at species or strain levels	Resolution is frequently not very good, especially in communities with higher diversity
TGGE (temperature gradient gel electrophoresis)	Yes	Differences among samples can be detected at species or strain levels	Resolution is frequently not very good, especially in communities with higher diversity. Special equipment making a temperature gradient is required
SSCP (single-strand conformation polymorphism)	Yes	Differences among samples can be detected at species or strain levels	Resolution is frequently not very good, especially in communities with higher diversity. Temperature-controlled electrophoresis is required
qRT-PCR (quantitative real-time polymerase chain reaction)	Yes	Quantification of specific genes	Specific primer set is required
SIP (stable isotope probing)	Yes (for combination with DGGE)/No	Active organisms can be analyzed with DNA or other stable compounds	Stable isotope of P is absent
Immunocapture using BrdU (5-bromo-2-deoxyuridine)	Sometimes yes	Active organisms can be analyzed. Immunoprecipitation can be applied	Dynamics of BrdU in soil are not well understood
Microarray	No	Whole aspects of specific microbes or mRNAs can be detected	Expensive. Universal microbe arrays are not yet applied
Next generation sequencer	No	Metagenomic and metatranscriptomic data are available	Very expensive
FISH (fluorescence in situ hybridization)	No	Localization of specific microbes or genes can be visualized	Amplification of signals is required for functional genes
PLFA (phospholipid fatty acid)	No	Microbial community structure can be analyzed independently from DNA or RNA	Lack of sequence information
Enzyme activities measured with fluorogenic substrates	No	Applicable for small amounts of soil samples	Dynamic range is different among the substrates dependent on activities
ELF (enzyme-labeled fluorescence)-97 phosphate	No	Localization of phosphatase	Application is specific
Phosphate-reporter bacteria	No	P availability for bacteria can be visualized	Application is specific

PCR polymerase chain reaction

organic compounds. Humic substances in particular, which behave like nucleic acids in some respects, cause problems during DNA extraction, purification, and further experimentation. Thus, the removal of humic substances and their separation from soil DNA are important steps.

Numerous methods for soil DNA extraction have been reported and several kits for soil DNA extraction are commercially provided, e.g., from Epicentre Biotechnologies (Madison, WI, USA), MO Bio Laboratories (Carlsbad, CA, USA), Nippongene (Toyama, Japan), Norgen Biotek (Thoroid, Canada), Omega Bio-Tek (Norcross, GA, USA), and Zymo Research (Orange, CA, USA). Currently, kits that include a bead-beating step are commonly used for soil DNA extraction. Because we mostly work with volcanic ash soils with high affinity to phosphates, we mainly employ the ISOIL kit from Nippongene, which is optimized for humic substance removal from volcanic ash soils (the method for removing the humic substances is protected by patent).

4.2.2 Plants and Hyphae

DNA extraction from plants is important for the investigation of the cooperation of plants with symbiotic and associated microbes in P cycling, as well as for the role of plants in these processes. Fungal DNA can be extracted by similar methods to those used for plants. We employ the CTAB (cetyltrimethylammonium bromide; a detergent for solubilizing membranes) method (Rogers and Bendich 1985) or commercially available kits such as the Plant DNA Mini Kit from Qiagen (Hilden, Germany) and Isoplant (Nippongene), for DNA extraction from plants and fungal mycelia. Grinding with pestle and mortar or bead-beating of samples frozen in liquid nitrogen is an effective preparation of tissues prior to DNA extraction. DNA extraction from plants or fungi is relatively easy because of the absence of humic substances.

In the case of arbuscular mycorrhizal (AM) fungi, spores can be collected from soils by sieving. This method enables DNA extraction specifically from spores of AM fungi. The yield of DNA is frequently very low; in such cases, addition of coprecipitating agents such as glycogen is recommended at the ethanol precipitation step.

4.2.3 Rolling Cycle Amplification

When rhizosphere soil or spores of AM fungi are targeted, the yield of DNA is sometimes too low (in the nanogram order) for direct analysis. Rolling cycle amplification (RCA) using Phi29 DNA polymerase can provide linearly amplified and purified DNA; commercial kits are available. In our experience, ca. 15 μg DNA can be amplified from 5 ng of DNA extracted from spores of *Gigaspora margarita*

by using GenomiPhi v2 (GE Healthcare, Buckinghamshire, UK). Amplified DNA can be used for further analyses, such as polymerase chain reaction (PCR)-dependent molecular tools and metagenomics.

4.3 Molecular Approaches Using the Sequences of SSU rRNA and ITS Regions

4.3.1 Clone Libraries

Small subunit (SSU) rRNA and internal transcribed spacer (ITS) regions are frequently analyzed for the molecular characterization of uncultured microbes. These sequences are easily amplified by PCR. Amplified fragments are compared with respect to their length, restriction fragment patterns, and substantial differences between sequences.

Sequencing of clone libraries is a simple method for the analysis of SSU rRNA or ITS regions. The sequence provides useful information on the phylogenetic position of the microbe. The ratio of overlap of sequences indicates the diversity of the group in the sample. Phylogenetic trees can be prepared based on the sequences. High-throughput analysis is possible using 96- or 384-hole multiplates. The disadvantage of this method is the relatively high expense.

Monteiro et al. (2009) analyzed partial 16S rRNA sequences for 83 bacterial colonies, which were isolated from the rhizosphere of vetiver (*Chrysopogon zizanioides*) as potential biofertilizers. A primer set for PCR-DGGE, which is described in the next section, was used for PCR and sequencing. It was revealed that potentially phosphate solubilizing bacteria coexisted in the vetiver rhizosphere that were closely related to *Acinetobacter*, *Burkholderia*, *Chryseobacterium*, *Dyella*, *Enterobacter*, *Klebsiella*, and *Pseudomonas*.

4.3.2 PCR-DGGE

Denaturant gradient gel electrophoresis (DGGE) is frequently applied for comparing the microbial communities of various environments (Heuer et al. 1997). The work-flow of DGGE is shown in Fig. 4.1. DNA fragments amplified from environmental DNA by PCR are separated on polyacrylamide gels containing gradients of denaturants. Differences of sequences (i.e., GC contents) result in different denaturing points, which show as bands on the denaturant gradient gel. A GC clamp (an artificial sequence rich in guanine and cytosine) that generates a tight bond is often added to the terminus of one primer to increase the resolution of the detected bands (Sheffield et al. 1989). The band patterns obtained by PCR-DGGE are used to

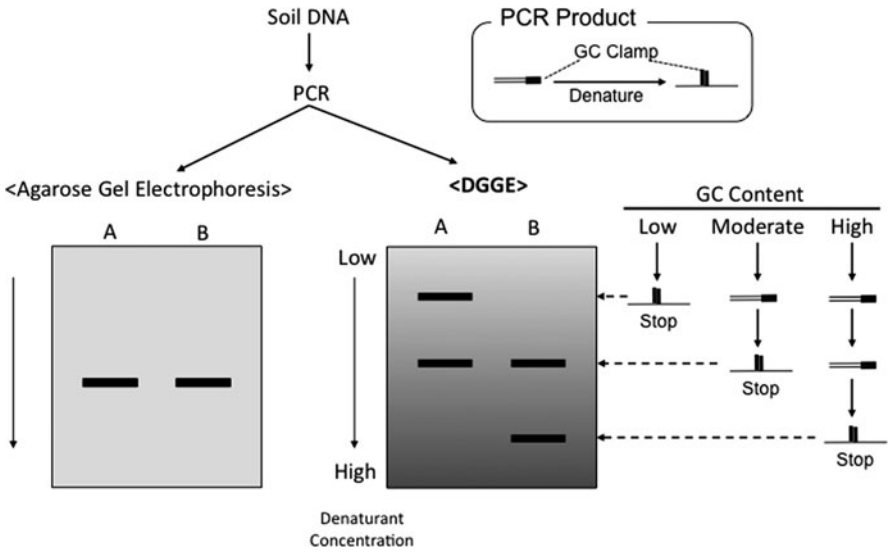

Fig. 4.1 The principle and work-flow of PCR-DGGE

compare samples by multivariate analyses such as principal component analysis or cluster analysis.

The merit of the DGGE method is the ease with which microbial communities of different samples can be compared without DNA sequence determination. The only technical requirement for DGGE is electrophoresis equipment. DGGE can visualize differences at the line or species levels, because bands can be differentiated even if the sequences compared differ merely in one base. By contrast, DGGE is unsuitable for the detection of differences at the genus or any higher levels. When the diversity of the sample is high, the bands separated in the DGGE are poorly resolved.

Marschner et al. (2004) applied PCR-DGGE of 16S rDNA to investigate microbial community structures of the rhizospheres of chickpea, canola, and Sudan grass as affected by P fertilization. Similarly, Wasaki et al. (2005) and Weisskopf et al. (2005) studied the community structures of rhizobacteria associated with white lupin plants, in order to clarify the effects of root exudates on the diversity and community structure of rhizobacteria in cluster roots formed by P-starved white lupin plants. Their results suggested that the diversity and community structure were strongly influenced by root exudates, such as citrate and flavonoids.

Temperature gradient gel electrophoresis (TGGE) resembles DGGE (Henco and Heibey 1990) in principle but differs in having a temperature rather than a denaturant gradient. Single-strand conformation polymorphism electrophoresis (SSCP) allows the separation of single-stranded DNA following the chilling-induced formation of secondary structures in the single-stranded DNA. Both TGGE and SSCP can separate sequences that differ in only one base. These techniques have not yet been applied in studies on soil P cycling.

4.3.3 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) visualizes microbial communities as patterns of restriction fragment length. RFLP targeted to SSU rRNA is designated as ARDRA (amplified ribosomal DNA restriction analysis; Massol-Deya et al. 1995). ARDRA has been used to characterize cultured isolates of potential biofertilizers (Monteiro et al. 2009). Because SSU rRNA is relatively short (>1,500 bp), its fragments are generally digested by a restriction enzyme that recognizes four bases. The restriction fragments are separated by electrophoresis. RFLP is a useful method for detecting relatively substantial differences (higher than genus level), but it cannot resolve small differences between the sequences compared. Specific bands can be isolated from gels and sequenced, although the sequence length is frequently too short for species identification.

T-RFLP (terminal RFLP) using a primer labeled with a fluorescent dye at the 5′-terminus is a useful technique for the detection of specific fragments. The fluorescence can be detected with high resolution by DNA sequencers or other analytic equipment. George et al. (2009) used T-RFLP to examine the effect of extracellular release of phytase from roots of transgenic plants on microbial community structures in the rhizosphere. They demonstrated that the expression of phytase in transgenic plants had little or no impact on the microbial community structure as compared with control plant lines.

4.4 Methods Targeting Active Microbes and Functional Genes

4.4.1 RNA Extraction

DNA work is relatively easy to apply on environmental samples, but it delivers information not only about active, but also about inactive microbes. Environmental RNA-targeted molecular approaches are required to target the major active players or key genes in the environment. However, RNA extraction from soil is still difficult because of the instability of RNA molecules, and only a few RNA-based studies have succeeded in identifying microorganisms involved in P cycling.

Weisskopf et al. (2005) investigated the bacterial community structures associated with white lupin, a plant that forms “cluster roots” showing an exudative burst under low P conditions, by producing DGGE profiles based on both DNA and RNA. In their study, RNA was isolated using a kit provided by BIO 101 (Vista, CA, USA). They showed that the “present” and “active” populations analyzed by DNA and RNA, respectively, were similar in the root samples but not in the rhizosphere and bulk soils. The effect of root type and age on the structure of bacterial communities living in the root vicinity was more obvious in active communities than in present communities. Thus, monitoring changes in active communities proved to be more informative than dealing only with present communities.

4.4.2 Analysis of Functional Genes by DNA-Based Techniques

Because soil RNA extraction is still problematic in many cases, the identification of functional genes involved in nutrient cycling so far mainly relies on DNA-based techniques.

We developed a PCR-DGGE methodology for the analysis of functional genes. Sakurai et al. (2008) designated specific primer sets for alkaline phosphatase (ALP), which is involved in organic P cycling. The primers were designed on the basis of an alignment of amino acid and nucleotide sequences encoding an ALP-expressing gene derived from various isolates: *Bacillus subtilis* 168, *Nostoc* sp. PCC7120, *Caulobacter crescentus* CB15, *Pseudomonas aeruginosa* PAO1, *Sinorhizobium meliloti* 1021, *Mesorhizobium loti* MAFF303099, and *Corynebacterium glutamicum* ATCC13032. Effects of the application of organic matter and chemical fertilizer on ALP-harboring bacterial communities in the rhizosphere and bulk soil in an experimental lettuce field were analyzed by PCR-DGGE. Numerous *ALP*-expression genes were detected in the DGGE profile, regardless of sampling time, fertilizer treatment, or sampled soil area, which indicated a large diversity in ALP-harboring bacteria in the soil. Several *ALP* gene fragments, which were excised from the DGGE gel and sequenced, were closely related to the ALP-expressing genes of *M. loti* and *Pseudomonas fluorescens*. Using principal component analysis of the DGGE profile it was shown that fertilizer treatment and sampling site significantly affected the community structures of ALP-harboring bacteria.

In the case of functional genes, the data obtained from the DGGE profile is expected to be quite informative. However, missing groups with a lower similarity of the respective functional gene to the sequence used for primer design is an undeniable possibility. For example, the identity of sequences among microorganisms was generally lower for *ALP* genes than for the sequences of SSU rRNA (Sakurai et al. 2008). Therefore, the two primer sets were designed for nested PCR of ALP gene fragments to increase the coverage range.

4.4.3 Quantitative Real-Time PCR: Quantification Technology for SSU rRNA and Transcripts of Functional Genes

Recently, the amount of mRNA in a wide range of samples has been analyzed by PCR-based methods rather than by hybridization-dependent methods. The merits of PCR-based methods are speed, reproducibility, and quantitative capacity. Quantitative RT-PCR (qRT-PCR), in particular, is very effective for microscale samples such as rhizosphere soils.

qRT-PCR for environmental DNA determines the quantity of microorganisms harboring the targeted gene. When genus-specific primers for SSU rRNA are used, the results of the technique can quantify the dominance of the genus. In the case of

RNA-based methods, the amount of mRNA of the target gene can be determined. The method is useful for functional genes that are directly involved in P cycling such as phosphatases, phytases, and nucleases. Melting curves of amplified fragments produced by most of the commercial equipments provide information on the diversity of the amplified fragments.

qRT-PCR was applied to evaluate interactions of arbuscular mycorrhizal fungi. Specific primers for ITS1 and rRNAs of *Glomus mosseae* and *G. intraradices* were designed for qRT-PCR (Alkan et al. 2006). These authors showed some effects of P availability in the soil on the interaction between the two *Glomus* species, and concluded that qRT-PCR was a valuable tool for studying the ecology of AM fungi.

4.4.4 Fluorescence In Situ Hybridization

The distribution of microbial species or functional groups of interest can be visualized by FISH (fluorescence in situ hybridization). A specific primer designed for SSU rRNA or a functional gene, and containing a fluorescent dye, is required for FISH. The dye must not interfere with the autofluorescence of soil particles and plant roots. The sample is hybridized with the dye primer and observed with a fluorescence microscope.

Usually, direct hybridization is possible for SSU rRNA because of the huge amount of molecules. On the other hand, amplification of the signal is required for most functional genes. Several methods have been developed such as MAR- (microautoradiography) (Ito et al. 2002), CARD- (catalyzed reporter deposition) (Pernthaler et al. 2002) and TSA (tyramide signal amplification)-FISH (Schriml et al. 1999). MAR-FISH was applied to study P cycling, even if not in soils. It was applied for the detection of potential polyphosphate-accumulating bacteria in biological phosphorus removal plants (Kong et al. 2005).

4.4.5 Stable Isotope Probing

Stable isotope probing (SIP) is a method for identifying active microbes in the environment. ^{13}C- or ^{15}N-labeled substrates are frequently used for SIP. For example, microbes that respond to root-secreted compounds can be detected in the rhizosphere of $^{13}CO_2$-assimilating plants. Rangel-Castro et al. (2005) determined the effect of liming on the structure of the rhizosphere microbial community metabolizing root exudates by the combination of DGGE and SIP. Phospholipid fatty acid analysis (PLFA) can be applied following SIP, and has been employed, e.g., for the analysis of methanotrophic communities (Chen et al. 2008).

Unfortunately, there are no useful stable isotopes of P. Thus, it seems difficult to apply SIP for studying P cycling. However, as mentioned by Prosser et al. (2006), SIP provides the potential for cultivation-independent characterization of

organisms actively assimilating carbon derived from plant root exudates or added to the soil. Since root exudates have impacts on P cycling in the rhizosphere, ^{13}C-SIP could be applied for studying the effects of root exudates on the microbes involved in P cycling.

4.4.6 Bromodeoxyuridine Immunocapture

An analog of thymidine, 5-bromo-2-deoxyuridine (BrdU), can be used for labeling active microorganisms in the environment. BrdU-antibodies are then applied to detect the microorganisms containing BrdU. Artursson et al. (2005) employed BrdU immunocapture in combination with T-RFLP. They found distinct changes in active bacterial community compositions related to *G. mosseae* inoculation, treatment with an antifungal compound, and plant type. The dominant bacterial species that were activated as a result of *G. mosseae* inoculation were found to be mostly uncultured bacteria and *Paenibacillus* species.

4.4.7 Phosphate-Reporter Bacteria

Phosphate-reporter bacteria have been developed and used for studies on P cycling. In particular, de Weger et al. (1994) developed phosphate-reporter strains of *Pseudomonas putida* WCS358 in which the production of β-glucosidase was regulated by the promoter of a gene responsive to P starvation. Kragelund et al. (1997) used two phosphate-reporter strains of *P. fluorescens* DF57 harboring luciferase regulated by the promoter of a gene responsive to P starvation. These phosphate-reporter bacteria were used to assess whether sufficient phosphate was available to the bacteria. It can be a useful tool for studying the plant–microbe interactions involved in P cycling, because the *Pseudomonas* spp. are frequently isolated as potentially plant growth-promoting rhizobacteria.

4.5 Methods for Analysis of Plant Functions

Root-secreted compounds of plants are important for P cycling in soil (Fig. 4.2). Acid phosphatase (APase) secreted from white lupin roots contributes most of the phosphatase activity in rhizosphere soil (Wasaki et al. 2005). Organic acids mobilize sparingly soluble P compounds in the soil. Mobilized inorganic phosphate can then be absorbed by plant roots with the help of phosphate transporters. Secondary plant metabolites are involved in the communication with rhizosphere microbes. Finally, mRNA expression of important genes in plants has been

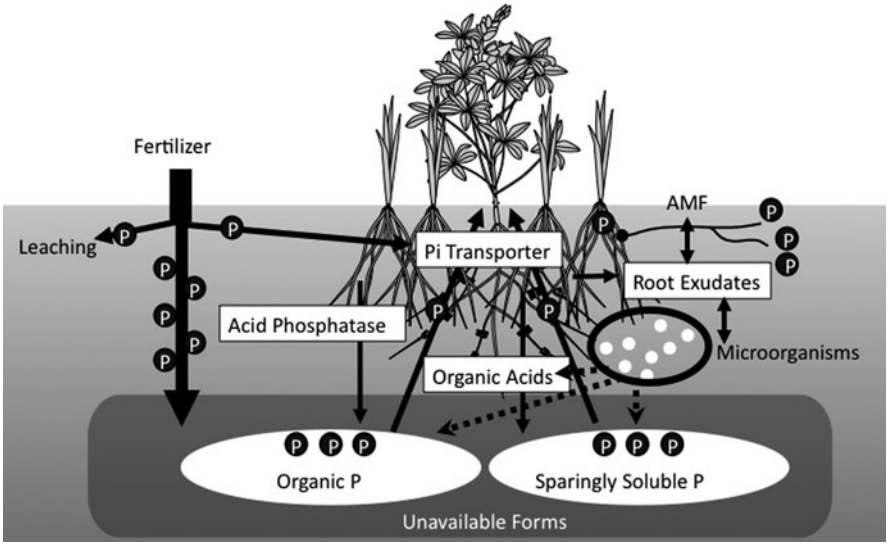

Fig. 4.2 Biological functions in the soil P cycle. *AMF* arbuscular mycorrhizal fungi

frequently analyzed by qRT-PCR. In this section, the methods for analysis of the above-mentioned plant functions involved in P cycling are introduced.

4.5.1 Phosphatase Activities

A method using *p*-nitrophenylphosphate (*p*-NPP) as a substrate is still applied for the measurement of phosphatase activity in soils and root exudates (Ozawa et al. 1995). The degradation product *p*-nitrophenol turns yellow following alkalinization. A fluorogenic substrate, 4-methylumbeliferylphosphate (MUP), is also available for the measurement of phosphatase activities. Many fluorogenic substrates can be used for the determination of soil enzyme activities involved in P, N, and C cycling. For high-throughput analysis of many soil enzyme activities in small samples, 96-well microplates and microplate readers are useful tools that allow detection of the fluorescence of the degradation products (Tscherko et al. 2004; Wasaki et al. 2005). For further detail on these assays see Nannipieri et al. (2011).

Phosphatase activity can be visualized by activity staining of phosphatases in the media or in gels after electrophoresis. For activity staining, the above-mentioned substrates (*p*-NPP and MUP) are often applied, for example in our studies (Wasaki et al. 1997, 1999, 2005). We have shown that the cluster roots of white lupin released a significant amount of APase under P-deficient conditions (Wasaki et al. 1999). Naphtylphosphate/fast-red TR (4-chloro-2-methylbenzenediazonium salt) can also be used for activity staining (Neumann and Martinoia 2002).

Enzyme-labeled fluorescence (ELF)-97 phosphate can be used for histochemical localization of phosphatases. This substrate provides fluorescent precipitates after

hydrolysis by phosphatases. We have shown strong APase activity in the epidermal tissues of normal roots and cluster rootlets and in root hairs of cluster rootlets under P deficiency using ELF-97 phosphate as a substrate (Wasaki et al. 2008). Actually, the strong activity in the rhizosphere of cluster roots formed by white lupin under P starvation was derived from root-secreted acid phosphatase (Wasaki et al. 2005). The gene for this root-secreted acid phosphatase was cloned and designated as *LASAP2* (Wasaki et al. 2000). *LASAP2* overexpressing tobacco lines were established and it was tested whether *LASAP2* contributed to P uptake from organic P in the soil (Wasaki et al. 2009). Our results suggest that P uptake by the transgenic plants increased both in aseptic gel media and under soil culture conditions.

4.5.2 Organic Acid Exudation from Plant Roots

Root-secreted organic acids have chelating abilities for metal ions in the soil, and mobilize P compounds bound to the metal ions or adsorbed to soil particles. Knowing the amount and composition of the secreted organic acids is important to better understand P mobilization in the rhizosphere. Rhizobox systems, developed by Dinkelaker and Marschner (1992), are useful for the localization of organic acids in roots. Organic acids trapped with clean filter paper can be measured by HPLC (high-performance liquid chromatography) or enzymatic quantification (Neumann and Römheld 1999; Wasaki et al. 2005). Commercial kits for enzymatic quantification of citrate and malate are available (e.g. Roche Diagnostics, Basel, Switzerland). For more details on P solubilization by organic acids see Jones and Oburger (2011).

4.5.3 Phosphate Transporters

Inorganic phosphate (Pi) is the form of P that is taken up by plants. Plants have two classes of Pi transporters, namely high- and a low-affinity types. Because the Pi concentration in the soil solution is quite low, it is believed that high-affinity Pi transporters play the major role in plant Pi uptake from the rhizosphere. In the case of *Arabidopsis*, there are nine members of high-affinity Pi transporters (Mudge et al. 2002). It was shown that AtPT1 is a high-affinity Pi transporter that cotransports H^+ by overexpression (Mitsukawa et al. 1997). It was indicated that one of the members, Pht1;4 (AtPT2), contributes greatly to phosphate uptake in P-deprived *Arabidopsis* (Misson et al. 2004).

Interestingly, the accumulation of mRNA for a high-affinity Pi transporter is synchronized with the infection of AM fungi (Nagy et al. 2005). Thus, it appears that the Pi transporter plays a role in Pi transport from arbuscules to plant cells. The accumulation of the mRNA for the low-affinity Pi transporter was not regulated by internal P levels of the plants. It appears likely that this type of transporter contributes to internal P translocation rather than P uptake.

4.5.4 Exudation of Antibiotic Compounds and Enzymes

White lupin has a high ability to mobilize P from sparingly soluble P through the secretion of organic acids (Gardner et al. 1983). The major root-secreted organic acid is citrate, which is readily consumed by microbes. To protect the secreted acids from degradation by microbes, white lupin secretes several compounds together with the organic acids (Weisskopf et al. 2006). Net proton release decreases the population of bacteria. Flavonoids and cell-wall-degrading enzymes protect the organic acids from consumption by fungi.

Metabolomic approaches can be useful for analysis of low molecular weight substances in root exudates (see Sect. 4.6.3). Several fluorogenic substrates can be applied for measurement of antibiotic enzyme activities (see Sect. 4.5.1).

It appears that plants and microorganisms compete for P (Unno et al. 2005). Thus, the secretion from roots of substances that protect secreted organic acids from degradation might be of general significance in soils. It seems necessary to consider the function of these antimicrobial substances in the context of plant–microbe interactions involved in P cycling.

4.5.5 Quantitative RT-PCR

qRT-PCR is frequently used for the quantification of plant mRNA, because it is a convenient and straightforward methodology, as discussed above. However, plants sometimes have numerous homologs of a given gene with different functions. Therefore, primers have to be designed carefully to detect a specific gene.

We have tried to analyze the metabolic alterations of P-deficient rice roots by transcriptomic analysis (see Sect. 4.6.1; Wasaki et al. 2003). It was indicated that two Pi transporters were regulated by P deficiency. qRT-PCR revealed that the Pi transporter expression found by cDNA microarray was exactly regulated by P deficiency.

4.6 Novel Technologies: Omics Analyses for P Cycling

4.6.1 Microarrays

The so-called omics analyses have become feasible during the last 10 years. Transcriptomics have been applied prior to other postgenomic strategies such as metabolomics and proteomics. Some transcriptomic studies of low-P adaptation strategies have been carried out using cDNA arrays for plants (Uhde-Stone et al. 2003; Wu et al. 2003; Hammond et al. 2003, 2004; Misson et al. 2005; Wang et al.

2002; Wasaki et al. 2003, 2006). Microarrays on a chip or a glass slide contain thousands of redundant sequences, which can be hybridized with fluorescent dye-labeled samples. The fluorescence is detected using specific scanners, which is the most important requirement. Some new important metabolic changes were suggested in our study of P-deficient rice roots, namely: (1) acceleration of carbon supply for organic acid synthesis through glycolysis; (2) alteration of lipid metabolism; (3) rearrangement of compounds for cell walls; and (4) changes in gene expression related to the response to metallic elements such as Al, Fe, and Zn (Wasaki et al. 2003).

Genomic sequencing or collections of expressed sequence tags are required before microarrays can be designed. Recently, ready-made arrays have become available not only for common model organisms such as *Arabidopsis* and rice, but also for woody plants including poplar, eucalyptus, and grape, and for crop species such as soybean, wheat, and maize.

Microbes involved in P cycling could be detected by the microarray technique if a microarray targeted for soil microbes is developed. He et al. (2007) developed a microarray designated the "GeoChip," containing 24,243 oligonucleotide probes and covering >10,000 genes in >150 functional groups involved in N, C, S, and P cycling, metal reduction and resistance, and organic contaminant degradation. Their array was successfully used for tracking the dynamics of metal-reducing bacteria and associated communities.

4.6.2 *Metagenomics and the Next Generation of DNA Sequencers*

To study uncultured microorganisms, environmental DNA can be recovered and sequenced. This approach is called "metagenomics." It is expected that metagenomics will provide the information required for understanding the whole microflora in the soil, the functions of the organisms involved (including P cycling), and the isolation of beneficial genes from uncultured microorganisms.

Recently, next-generation DNA sequencers have been developed (Mardis 2008). This development will contribute to progress not only in metagenomics but will also enable metatranscriptomic studies on P cycling. Furthermore, the method will facilitate transcriptomic analyses of not-yet sequenced organisms.

Recently, Badri et al. (2009) reported the first study on the microbial community in the rhizosphere using a pyrosequencer, one of the next-generation DNA sequencers. A metagenomic approach revealed that exudates from an *Arabidopsis* mutant of the ATP-binding cassette (ABC) transporter involved in root secretion of phytochemicals cultivated a microbial community with a relatively greater abundance of potentially beneficial bacteria. The next generation of DNA sequencers could support metagenomic and metatranscriptomic studies on P cycling.

Table 4.2 Application of molecular tools for studies on P cycling

Question	Target molecules	Molecular tools	Examples of application for studying P cycling
Structure of microbial communities involved in P cycling	SSU rRNA	Clone library	Monteiro et al. (2009)
		DGGE/TGGE/SSCP	Marschner et al. (2004), Wasaki et al. (2005), Weisskopf et al. (2007)
		RFLP	George et al. (2009), Monteiro et al. (2009)
		Microarray	–
	Phospholipid fatty acids	PLFA	Tscherko et al. (2004)
Active microbes involved in P cycling	Functional genes	Clone library	–
		DGGE/TGGE/SSCP	Sakurai et al. (2008)
		RFLP	–
	Metagenomics	Next generation sequencer	Badri et al. (2009)
Quantitative analysis of specific microbes	SSU rRNA/functional genes	qRT-PCR	Alkan et al. (1995)
Localization of specific microbes	SSU rRNA/functional genes	FISH	–
P availability in the rhizosphere	P starvation responsive gene	Phosphate-reporter bacteria	de Weger et al. (1994), Kragelund et al. (1997)
Enzyme activities involved in P cycling	Enzyme activities	Fluorogenic substrates	Tscherko et al. (2004), Wasaki et al. (2005)
Localization of phosphatase activities	Phosphatase	ELF-97 phosphate	Wasaki et al. (2008)
Response of microbes to root exudates	SSU rRNA/functional genes	SIP-DGGE	–
	Phospholipid fatty acids	SIP-PLFA	–
Active microbes involved in P cycling	SSU rRNA/functional genes	BrdU	Artursson et al. (2005)
Plant responses to P deficiency	Transcriptomics	Microarray	Hammond et al. (2003, 2004), Uhde-Stone et al. (2003), Wu et al. (2003), Wang et al. (2002), Misson et al. (2005), Wasaki et al. (2003, 2006)
		Next generation sequencer	–
Effects of root-secreted enzymes	Phosphatase/phytase	Overexpression	Wasaki et al. (2008)

4.6.3 *Proteomics and Metabolomics*

Methods for proteomics and metabolomics became available due to the development of mass spectrometric techniques such as TOF (time of flight)-MS (mass spectrometry), GC (gas chromatography)-MS/MS, and LC (liquid chromatography)-MS/MS. These will also contribute to the understanding of P cycling. A proteomic study on P- and Al-stressed rice plants has already been reported (Fukuda et al. 2007). Crude proteins of control and stressed roots were separated on two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and the unique spots found in samples from stressed plants were determined by peptide mapping using TOF-MS. It was shown that the proteomic alterations under P deficiency and Al stress conditions were similar, indicating that a common metabolic system is responsive to both P deficiency and Al stress.

Suzuki et al. (2009) reported the metabolomic analysis of root exudates of rice seedlings. GC-MS was used for the analysis of primary metabolites in the study. Their results suggested that the physiological change during seedling development was large and that the response to the environment was rather small. We expect that further improvement of these methods and the combination of molecular approaches and metabolomics will provide crucial information.

4.7 Conclusions

In Table 4.2, we have summarized the potential applications of molecular tools for studying P cycling, with examples from the literature. The relationships between tools, samples, and targets are illustrated in Fig. 4.3. We would be happy if this chapter helps readers' studies on P cycling.

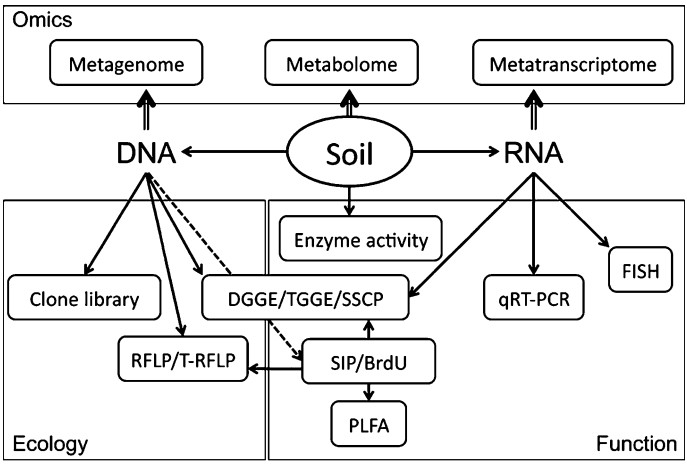

Fig. 4.3 Molecular approaches for analyzing the ecology and function of soil microorganisms

Molecular approaches and recent omics analyses have proven powerful tools for clarifying processes and actors involved in P cycling. On the other hand, we must not forget the advantages of conventional, culture-dependent methods. For example, metagenomic data cannot identify intracellular symbioses. The "living" cell is the central focus of microbial ecology. Adequate molecular approaches have to be selected that provide meaningful complements to conventional approaches for future investigations into P cycling.

References

Alkan N, Gadkar V, Yarden O, Kapulnik Y (2006) Analysis of quantitative interactions between two species of arbuscular mycorrhiz fungi, *Glomus mosseae* and *G. intraradices*, by real-time PCR. Appl Environ Microbiol 72:4192–4199

Artursson V, Finlay RD, Jansson JK (2005) Combined bromodeoxyuridine immunocapture and terminal-restriction fragment length polymorphism analysis highlights differences in the active soil bacterial metagenome due to *Glomus mosseae* inoculation or plant species. Environ Microbiol 7:1952–1966

Badri DV, Quintana N, El Kassis EG, Kim HK, Choi YH, Sugiyama A, Verpoorte R, Martinoia E, Manter DK, Vivanco JM (2009) An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol 151:2006–2017

Chen Y, Dumont MG, McNamara NP, Chamberlain PM, Bodrossy L, Stralis-Pavese N, Murrell JC (2008) Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. Environ Microbiol 10:446–459

de Weger LA, Dekkers LC, van der Bij AJ, Lugtenberg BJJ (1994) Use of phosphate-reporter bacteria to study phosphate limitation in the rhizosphere and in bulk soil. Mol Plant Microb Interact 7:32–38

Dinkelaker B, Marschner H (1992) In vivo demonstration of acid-phosphatase-activity in the rhizosphere of soil-grown plants. Plant Soil 144:199–205

Fukuda T, Saito A, Wasaki J, Shinano T, Osaki M (2007) Metabolic alterations proposed by proteome in rice roots grown under low P and high Al concentration under low pH. Plant Sci 172:1157–1165

Gardner WK, Barber DA, Parbey DG (1983) The acquisition of phosphorus by *Lupinus albus* L. III. The probable mechanism by which phosphorus movement in the soil/root interface is enhanced. Plant Soil 70:107–124

George TS, Richardson AE, Li SS, Gregory PJ, Daniell TJ (2009) Extracellular rerelase of a heterologous phytase from roots of transgenic plants: does manipulation of rhizosphere biochemistry impact microbial community structure? FEMS Microbiol Ecol 70:433–445

Hammond JP, Bennett MJ, Bowen HC, Broadley MR, Eastwood DC, May ST, Rahn C, Swarup R, Woolaway KE, White PJ (2003) Changes in gene expression in Arabidopsis shoots during phosphate starvation and the potential for developing smart plants. Plant Physiol 132:578–596

Hammond JP, White PJ, Broadley MR (2004) Diagnosing phosphorus deficiency in plants. Asp Appl Biol 72:89–98

He Z, Gentry TJ, Schadt CW, Wu L, Liebich J, Chong SC, Huang Z, Wu W, Gu B, Jardine P, Criddle C, Zhou J (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME J 1:67–77

Henco K, Heibey M (1990) Quantitative PCR – the determination of template copy numbers by temperature-gradient gel-electrophoresis (TGGE). Nucleic Acid Res 18:6733–6734

Heuer H, Krsek M, Baker P, Smalla K, Wellington EMH (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63:3233–3241

Ito T, Nielsen JL, Okabe S, Watanabe Y, Nielsen PH (2002) Phylogenetic identification and substrate uptake patterns of sulfate-reducing bacteria inhabiting an oxic-anoxic sewer biofilm determined by combining microautoradiography and fluorescent in situ hybridization. Appl Environ Microbiol 68:356–364

Jones DL, Oburger E (2011) Solubilization of phosphorus by soil microorganisms. In: Bünemann EK, Oberson A, Frossard E (eds) Phosphorus in action: biological processes in soil phosphorus cycling, vol 26, Soil biology. Springer, Heidelberg, Berlin. doi:10.1007/978-3-642-15271-9_7

Kong YH, Nielsen JL, Nielsen PH (2005) Identity and ecophysiology of uncultured actinobacterial polyphosphate-accumulating organisms in full-scale enhanced biological phosphorus removal plants. Appl Environ Microbiol 71:4076–4085

Kragelund L, Hosbond C, Nybroe O (1997) Distribution of metabolic activity and phosphate starvation response of *lux*-tagged *Pseudomonas fluorescens* reporter bacteria in the barley rhizosphere. Appl Environ Microbiol 63:4920–4928

Mardis ER (2008) The impact of next-generation sequencing technology on genetics. Trends Genet 24:133–141

Marschner P, Crowley D, Yang CH (2004) Development of specific rhizosphere bacterial communities in relation to plant species, nutrition and soil type. Plant Soil 261:199–208

Massol-Deya AA, Odelson DA, Hickey RF, Tiedje JM (1995) Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In: Akkermans ADL, van Elsas JD, Bruijin FJ (eds) Molecular microbial ecology. Kluwer, Dordrecht, pp 1–8

Misson J, Thibaud MC, Bechtold N, Raghothama K, Nussaume L (2004) Transcriptional regulation and functional properties of Arabidopsis Pht1;4, a high affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. Plant Mol Biol 55:727–741

Misson J, Raghothama KG, Jain A, Jouhet J, Block MA, Bligny R, Ortet P, Creff A, Somerville S, Rolland N, Doumas P, Nacry P, Herrerra-Estrella L, Nussaume L, Thibaud M-C (2005) A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation. Proc Natl Acad Sci USA 102:11934–11939

Mitsukawa N, Okumura S, Shirano Y, Sato S, Kato T, Harashima S, 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 USA 94:7098–7102

Monteiro JM, Vollú RE, Coelho MRR, Alciano CS, Blank AF, Seldin L (2009) Comparison of the bacterial community and characterization of plant growth-promoting rhizobacteria from different genotypes of *Chrysopogon zizanioides* (L.) Roberty (Vetiver) rhizospheres. J Microbiol 47:363–370

Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in Arabidopsis. Plant J 31:341–353

Nagy R, Karandashov V, Chague W, Kalinkevich K, Tamasloukht M, Xu GH, Jakobsen I, Levy AA, Amrhein N, Bucher M (2005) The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. Plant J 42:236–250

Nannipieri P, Giagnoni L, Landi L, Renella G (2011) Role of phosphatase enzymes in soil. In: Bünemann EK, Oberson A, Frossard E (eds) Phosphorus in action: biological processes in soil phosphorus cycling, vol 26, Soil biology. Springer, Heidelberg, Berlin. doi:10.1007/978-3-642-15271-9_9

Neumann G, Martinoia E (2002) Cluster roots – an underground adaptation for survival in extreme environments. Trends Plant Sci 7:162–167

Neumann G, Römheld V (1999) Root excretion of carboxylic acids and protons in phosphorus-deficient plants. Plant Soil 211:121–130

Ozawa K, Osaki M, Matsui H, Honma M, Tadano T (1995) Purification and properties of acid phosphatase secreted from lupin roots under phosphorus-deficiency conditions. Soil Sci Plant Nutr 41:461–469

Pernthaler A, Pernthaler J, Amann R (2002) Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl Environ Microbiol 68:3094–3101

Prosser JI, Rangel-Castro JI, Killham K (2006) Studying plant-microbe interactions using stable isotope technologies. Curr Opin Biotechnol 17:98–102

Rangel-Castro JI, Killham K, Ostle N, Nicol GW, Anderson IC, Scrimgeour CM, Ineson P, Meharg A, Prosser JI (2005) Stable isotope probing analysis of the influence of liming on root exudate utilization by soil microorganisms. Environ Microbiol 7:828–838

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh herbarium and minifield plant tissues. Plant Mol Biol 5:69–76

Sakurai M, Wasaki J, Tomizawa Y, Shinano T, Osaki M (2008) Analysis of bacterial communities on alkaline phosphatase gene in organic matter applied soil. Soil Sci Plant Nutr 54:62–71

Schriml LM, Padilla-Nash HM, Coleman A, Moen P, Nash WG, Menninger J, Jones G, Ried T, Dean M (1999) Tyramide signal amplification (TSA)-FISH applied to mapping PCR-labeled probes less than 1 kb in size. Biotechniques 27:608

Sheffield VC, Cox DR, Lerman LS, Myers RM (1989) Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. Proc Natl Acad Sci USA 86:232–236

Suzuki K, Okazaki K, Tawaraya K, Osaki M, Shinano T (2009) Gas chromatography-mass spectrometry associated grobal analysis of rice root exudates under aseptical conditions. Soil Sci Plant Nutr 55:505–513

Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245

Tscherko D, Hammesfahr U, Marx MC, Kandeler E (2004) Shifts in rhizosphere microbial communities and enzyme activity of *Poa alpina* across an alpine chronosequence. Soil Biol Biochem 36:1685–1698

Uhde-Stone C, Zinn KE, Ramirez-Yáñez M, Li A, Vance CP, Allan DL (2003) Nylon filter arrays reveal differential gene expression in proteoid roots of white lupin in response to phosphorus deficiency. Plant Physiol 131:1064–1079

Unno Y, Okubo K, Wasaki J, Shinano T, Osaki M (2005) Plant growth promotion abilities and micro-scale bacterial dynamics in the rhizosphere of lupin analyzed by phytate utilization ability. Environ Microbiol 7:396–404

Wang Y-H, Gravin DF, Kochian LV (2002) Rapid induction of regulatory and transporter genes in response to phosphorus, potassium, and iron deficiencies in tomato roots. Evidence for cross talk and root/rhizosphere-mediated signals. Plant Physiol 130:1361–1370

Wasaki J, Ando M, Ozawa K, Omura M, Osaki M, Ito H, Matsui H, Tadano T (1997) Properties of secretory acid phosphatase from lupin roots under phosphorus-deficient conditions. Soil Sci Plant Nutr 43:981–986

Wasaki J, Omura M, Ando M, Shinano T, Osaki M, Tadano T (1999) Secreting portion of acid phosphatase in roots of lupin (*Lupinus albus* L.) and a key signal for the secretion from the roots. Soil Sci Plant Nutr 45:937–945

Wasaki J, Omura M, Ando M, Dateki H, Shinano T, Osaki M, Ito H, Matsui H, Tadano T (2000) Molecular cloning and root specific expression of secretory acid phosphatase from phosphate deficient lupin (*Lupinus albus* L.). Soil Sci Plant Nutr 46:427–437

Wasaki J, Yonetani R, Kuroda S, Shinano T, Yazaki J, Fujii F, Shimbo K, Yamamoto K, Sakata K, Sasaki T, Kishimoto N, Kikuchi S, Yamagishi M, Osaki M (2003) Transcriptomic analysis of metabolic changes by phosphorus stress in rice plant roots. Plant Cell Environ 26:1515–1523

Wasaki J, Rothe A, Kania A, Neumann G, Römheld V, Shinano T, Osaki M, Kandeler E (2005) Root exudation, P acquisition and microbial diversity in the rhizosphere of *Lupinus albus* as affected by P supply and atmospheric CO_2 concentration. J Environ Qual 34:2157–2166

Wasaki J, Shinano T, Onishi K, Yonetani R, Yazaki J, Fujii F, Shimbo K, Ishikawa M, Shimatani Z, Nagata Y, Hashimoto A, Ohta T, Sato Y, Miyamoto C, Honda S, Kojima K, Sasaki T, Kishimoto N, Kikuchi S, Osaki M (2006) Transcriptomic analysis indicates putative metabolic changes caused by manipulation of phosphorus availability in rice leaves. J Exp Bot 57:2049–2059

Wasaki J, Kojima S, Maruyama H, Haase S, Osaki M, Kandeler E (2008) Localization of acid phosphatase activities in roots of white lupin plants grown under phosphorus-deficient conditions. Soil Sci Plant Nutr 54:95–102

Wasaki J, Maruyama H, Tanaka M, Yamamura T, Dateki H, Shinano T, Ito S, Osaki M (2009) Overexpression of *LASAP2* gene for secretory acid phosphatase of white lupin improves P uptake and growth of tobacco plants. Soil Sci Plant Nutr 55:107–113

Weisskopf L, Fromin N, Tomasi N, Arango M, Martinoia E (2005) Secretion activity of white lupin's cluster roots influences bacterial abundance, function and community structure. Plant Soil 268:181–194

Weisskopf L, Abou-Mansour E, Fromin N, Tomasi N, Santelia D, Edelkott I, Neumann G, Aragno M, Tabacchi R, Martinoia E (2006) White lupin has developed a complex strategy to limit microbial degradation of secreted citrate required for phosphate acquisition. Plant Cell Environ 29:919–927

Wu P, Ma L, Hou X, Wang M, Wu Y, Liu F, Deng XW (2003) Phosphate starvation triggers distinct alterations of genome expression in Arabidopsis roots and leaves. Plant Physiol 132:1260–1271