Doctoral Thesis

Study on Organic Acid Transporters Induced by Phosphorus Deficiency and Mobilization of Unavailable Phosphate in Soil by Root Exudates

(植物根におけるリン欠乏誘導型有機酸トランスポーターの同定と
分泌物による土壌中難利用性リン可給化機構の解明)

Hayato Maruyama

Department of Environmental Dynamics and Management
Graduate School of Biosphere Science
Hiroshima University

March 2012

Contents

Abbreviations

ALMT	aluminum activated malate transporter
APase	acid phosphatase
cDNA	complementary DNA
CE-TOF MS	capillary electrophoresis/time-of-flight mass spectrometer
CS	citrate synthase
DGGE	denaturing gradient gel electrophoresis
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
GC-MS	gas chromatograph/mass spectrometry
GFP	green fluorescent protein
GUS	ß-glucuronidase
ICDH	isocitrate dehydrogenase
MATE	multidrug and toxin extrusion
MES	2-(N-morpholino)-ethanesulfonic acid
P	phosphorus
PAP	purple acid phosphatase
PCR	polymerase chain reaction
PIP	plasma membrane intrinsic protein
p-NPP	*p*-nitrophenyl phosphate
RT-PCR	reverse transcription PCR
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
UNFPA	the United Nations Population Fund

Chapter 1

General introduction

1. Phosphorus resource and food production

The world has reached 7 billion people in October 2011, and it is estimated that another billion will added to our numbers by 13 years from now (UNFPA, http://www.unfpa.org/public/). Therefore, development of sustainable food production systems is critically important to avoid famine or health deterioration.

Phosphorus (P) is one of the major mineral nutrients required by plants. Owing to the strong reactivity of phosphates with soil minerals, P is largely unavailable to plants. Furthermore, P is exported from the field in the harvested products. The addition of P fertilizers to sustain crop production is thus required. However, unfortunately, P fertilizers are manufactured from nonrenewable resources that are increasingly becoming more costly and less available. It was estimated that mined rock phosphate reserves could easily be depleted by 2060 (Steen 1998, Vance et al. 2003). On the basis of an assumption that the increasing rate of phosphate rock production from 2009 to 2010 will continued in the future, the phosphate rock reserves will be depleted at 53 years from 2011 (Table 1-1). Especially, the increase of phosphate rock consumption is huge in China, ca. 3 times increased during this decade (US Geological Survey, http://www.usgs.gov/). As the result of the excess amount of P application in China, P accumulation is still progressed in the arable lands (Li et al. 2011).

Paradoxically, part of the applied P in intensive cropping systems can enter the waterways through runoff and erosion, contributing to pollution of surrounding lakes and marine environments. To solve the environmental problems

and to achieve the sustainable agriculture system, it is concluded that the utilization of accumulated P in soils is very important. That is to say, it is important to understand and apply strategies of crop plants to mobilize unavailable P in soils.

Table 1-1 Production and probable reserves of rock phosphate and life of phosphate rock estimated by the data from US Geological Survey, 2011 among main country and region of the world.

Country or region	Production of 2010 (million tons)	Probable reserves (million tons)	Life of phosphate rock resources (year)	
			No increase	Rate of increase in 2010
China	6.50	370	57	22
US	2.61	140	54	84
Morocco and Western Sahara	2.60	5,000	1923	63
Russia	1.00	130	130	>100
Tunisia	0.76	10	13	25
Jordan	0.60	150	250	>100
Brazil	0.55	34	62	27
Israel	0.30	18	60	19
Syria	0.28	180	643	35
South Africa	0.23	150	652	>100
Togo	0.08	6	75	>100
Senegal	0.07	18	277	>100
Other Countries	1.15	282	245	12
Total	17.60	6,500	370	53

2. Plant responses to P-deficient condition

Plants acquire P as the inorganic phosphate ion (Pi) through Pi transporters on plasma membranes of roots. However, the availability of Pi is often limited, because Pi can easily bind and precipitate with metal cations such as Al, Fe and Ca, and alter into organic compounds in soils (Raghothama 1999). The organic P generally makes up 30 - 80% of the total P in soil, and the predominant form of organic P is phytate, which composes up to 50% of soil organic P (Turner et al. 2008).

To avoid the limited availability of Pi, plants have evolved a repertoire of adaptive responses, which involve diverse developmental and biochemical processes, to increase Pi acquisition and utilization (Raghothama 1999). White lupin (*Lupinus albus* L.) has been well studied as a model of tolerant to

P-deficiency harboring various strategies to acquire P from soils. For example, mobilization of organic P and sparingly soluble P by secretion of organic acids and phosphatases (Ozawa et al. 1995; Gilbert et al. 1999; Neumann et al. 1999, 2000), modification of root architecture (Williamson et al. 2001), induction of H^+-ATPase and subsequent secretion of protons (Yan et al. 2002), and exudation of flavonoids (Weisskopf et al. 2006) (Fig. 1-1).

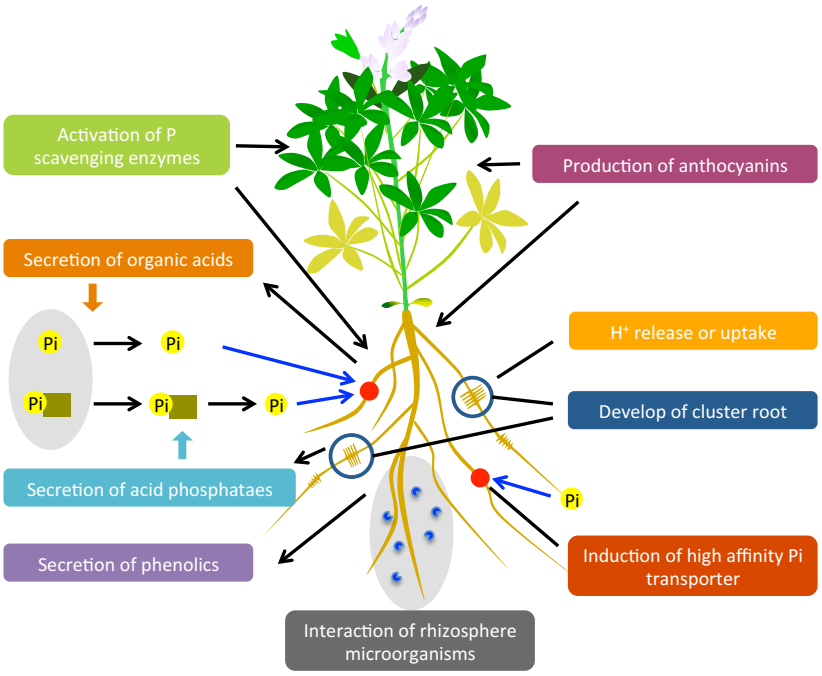

Figure 1-1 Morphological, physiological and molecular response of white lupin to P deficiency.

For mobilizing P in soil, it has been known that white lupin has a high ability to secrete organic acids and acid phosphatase (APase) from roots under P deficient condition (Gilbert et al. 1999; Neumann et al. 1999, 2000). White lupin forms unique root clusters like bottle blushes, so-called 'cluster roots' (or proteoid roots, because the typical roots are found in Proteaceae roots), when it is grown under poor conditions in nutrients such as P and Fe (Neumann and Martinoia 2002). It was shown that the cluster roots formed under P deficient conditions had specific ability to obtain P by not only the increase of root surface area but also the acquisition ability by increase of Pi transporters and exudation of organic acids and APases (Liu et al. 2001; Miller et al. 2001; Wasaki et al. 2003, 2008).

3. Roles of organic acids and APases secreted from plant roots and utilization of the functions by gene modification

Not only the lupin, most plants respond to P deficiency by secretion of organic acids and APases from roots (e.g. Hoffland et al. 1989; Jones 1998). Therefore, it is believed that secretion of organic acids and APases from roots is the important response to P deficiency for mobilizing soil P (Fig.1-2). However, the detail of the mechanisms is not clarified yet. In case of organic acid secretion, the transporters of organic acids responding to P deficiency are still unveiled.

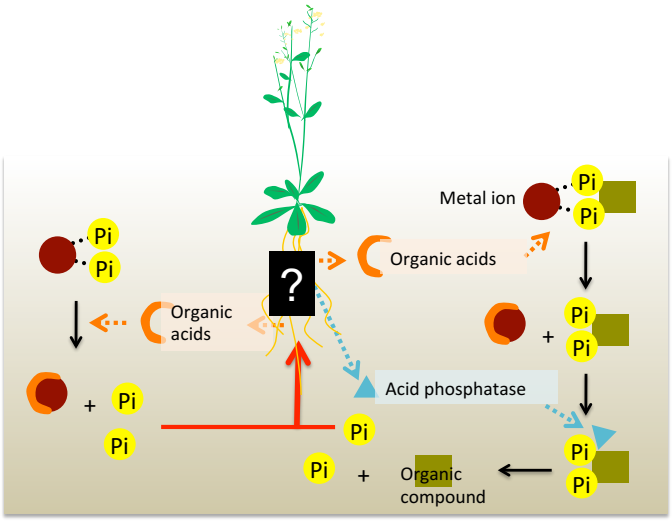

Figure 1-2 Roles of organic acids and acid phosphatases secreted from root P deficient soils.

Organic acids are known to compete with Pi for the same sorption sites (ligand exchange) or solubilize Pi via ligand promoted mineral dissolution. Under P deficient condition, it has been considered that citrate, malate, oxalate and acetate are major organic acids secreted from plant roots (Jones 1989). The extraction efficiency of inorganic P by the organic acids appears to follow series; citrate > oxalate > malate > acetate (Lan et al. 1995).

Organic acid secretion from plant roots into the rhizosphere plays an important role also in Al detoxification (Raghothama 1999; Vance et al. 2003; Ma et al. 2001; Ryan et al. 2001; Kochian et al. 2004). The mechanism of Al detoxification by organic acid secretion has been well investigated and it was known some important genes, such as wheat (*Triticum aestivum* L.) aluminum-activated malate transporter; *TaALMT1* (Sasaki et al. 2004) and Al inducible multidrug and toxin extrusion (MATE) family citrate transporters; *HvAACT1* (Furukawa et al. 2007) and

SbMATE (Magalhaes et al. 2007) from barley (*Hordeum vulgare* L.) and sorghum (*Sorghum bicolor* L.), respectively.

There are some studies to improve organic acid secretion from roots by modification of genes involved in the metabolisms of organic acids such as citrate synthase (CS) and isocitrate dehydrogenase (ICDH) (López-Bucio et al. 2000; Kihara et al. 2005). López-Bucio et al. (2000) reported that P accumulation of tobacco was improved as the result of CS overproduction. However, Delhaize et al. (2001) reported that transgenic tobacco plants overexpressing CS increased there protein but it did not show increased accumulation of citrate in roots or increased Al-activated efflux of citrate from roots. On the other hands, transgenic barley expressing the TaALMT1 shows enhanced the ability to acquire P from an acid soil (Delhaize et al. 2009). Thus, it is important not only modification of the genes involved in organic acids metabolisms but also transporter itself. Ma (2005) suggested that the molecular mechanisms of malate exudation are different between Al-stress and P-deficiency. Considering these reports, a hypothesis is raised that any homologs of ALMT and/or MATE family genes are involved in malate and citrate transport under P deficient conditions.

APases comprise a family of metal-containing glycoproteins that catalyze the hydrolysis of a wide range of phosphate esters and anhydrides. Many plant APase genes, such as *LePS2* from tomato (*Solanum lycopersicum* L.) (Baldwin et al. 2001, 2008), *StPAPs* from potato (*Solanum tuberosum* L.) (Zimmermann et al. 2003), and *MtPAP1* from *Medicago truncatula* (Xiao et al. 2006), were isolated and characterized. These APases are thought to play a role in the mobilization of Pi from organic P sources in the rhizosphere and in Pi scavenging (Duff et al. 1994). There were many studies to engineer crop plants improved mobilization of organic P from soils. Gene introduction of phytases was the main strategy of this, because the main form of organic P is phytate; such as *phyA* of *Aspergillus niger* (George et al. 2004, 2005b; Mudge et al. 2003; Richardson et al. 2001), *168phyA* of *Bacillus subtilis* (Yip et al. 2003), synthetic gene for secretory phytase (Zimmermann et al.

2003), and *MtPHY1* of *Medicago truncatula* (Xiao et al. 2005). Gene modification for non-specific APases was also tried, such as *MtPAP1* of *Medicago truncatula* (Xiao et al. 2006) and *LASAP2* of white lupin (Wasaki et al. 2009) (Table 1-2). Most of them concluded that these transgenic plants have improved ability to acquire P from phytate when grown in sterile agar, but show limited contributions to P uptake when grown in soil. It was considered that the low solubility of phytate-P in soils was restricted the efficiency of phytate-P utilization in soils (George et al. 2004).

When crude APase collected from white lupin exudates was injected into the rhizospheres of tomato and sugar beet plants, growth and P absorption of these plants increased (Tadano and Komatsu 1994). Ozawa et al. (1995) purified and characterized the APase protein, and the cDNA has been isolated and designated as *LASAP2* (Wasaki et al. 2000). Then, *LASAP1* and *LASAP3*, homologs of *LASAP2*, were isolated from P deficiency roots and germinating seeds of white lupin, respectively (Wasaki et al. 1999b, 2003). LASAP2 has wide substrate specificity, and is stable at pH 4.0 – 9.0 (Ozawa et al. 1995). Like many other secreted proteins, APase is glycosylated, which protects it against proteolytic enzymes and contributes to its stability (Ozawa et al. 1995). Deduced amino acid sequence of LASAP3 was highly identical (82%) with a phytase from germinating seeds of soybean, GmPhy7 (Hegeman and Grabau 2001), although the similarity of the sequence of LASAP3 was not very high with LASAP1 and LASAP2 (23 and 30%, respectively). It was shown that a recombinant protein of LASAP3 produced in *Escherichia coli* exhibited phytase activity with a *Km* value = 83.1 μM at pH 5.5 (Kaneko 2004). The sequence similarities and in vitro activity all strongly support the notion that LASAP3 also has phytase activity in white lupin plants. Therefore, it is believed that the LASAP2 and LASAP3 have a potential for utilization of organic P in soil, although it is required to investigate the expression properties of *LASAP3*.

Table 1-2 List of studies to engineer crop plants improved mobilization of organic P in soils.

Authors	Gene	Gene introduced	Promoter	Transformed plant species
Park et al. (1994)	pho5	Yeast	CaMV 35S	Tobacco
Richardson et al. (2001)	phyA	*Aspergillus niger*	CaMV 35S	Arabidopsis
	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	CaMV 35S	Arabidopsis
Mudge et al. (2003)	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	CaMV 35S	Arabidopsis
	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	Pht1;2	Arabidopsis
Yip et al. (2003)	168phyA	*Bacillus subtilis*	CaMV 35S	Tobacco
Zimmermann et al. (2003)	PHY	Synthesized	LeExt1.1	Potato
George et al. (2004)	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	CaMV 35S	Subterranean Clover
George et al. (2005)	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	CaMV 35S	Tobacco
Lung et al. (2005)	Cex::168phyA	*Bacillus subtilis* (Fusion Protein with Signal Peptide of Carrot Extensin)	CaMV 35S	Tobacco
	Aex::168phyA	*Bacillus subtilis* (Fusion Protein with Signal Peptide of Arabidopsis Extensin)	CaMV 35S	Tobacco
	Aex::168phyA	*Bacillus subtilis* (Fusion Protein with Signal Peptide of Arabidopsis Extensin)	CaMV 35S	Arabidopsis
Xiao et al. (2005)	MtPAP1	*Medicago truncatula*	MtPAP1	Arabidopsis
	sp'MtPAP1	*Medicago truncatula* (Fusion Protein with Signal Peptide of Potato Patatin)	MtPAP1	Arabidopsis
	sp'MtPAP1	*Medicago truncatula* (Fusion Protein with Signal Peptide of Potato Patatin)	CaMV 35S	Arabidopsis
Xiao et al. (2006)	MtPHY1	*Medicago truncatula*	CaMV 35S	Arabidopsis
	MtPHY1	*Medicago truncatula*	MtPT1	Arabidopsis
Hur et al. (2007)	OsACP1	Rice	CaMV 35S	Arabidopsis
Wasaki et al. (2009)	LASAP2	White Lupin	CaMV 35S	Tobacco
Ma et al. (2009)	MtPHY1	*Medicago truncatula*	CaMV 35S	White clover
	MtPHY1	*Medicago truncatula*	MtPT1	White clover
	MtPAP1	*Medicago truncatula*	CaMV 35S	White clover
	MtPAP1	*Medicago truncatula*	MtPT1	White clover
Li et al. (2009)	AfphyA	Aspergillus ficum	Atpylk10	Soybean
Liu et al. (2011)	ex::phyA	*Aspergillus niger* (Fusion Protein with Signal Peptide of Carrot Extensin)	Atpyk10	Cotton

4. Hypotheses and aims of this study

The theme of my study is 'Study on Organic Acid Transporters Induced by Phosphorus Deficiency and Mobilization of Unavailable Phosphate in Soil by Root Exudates'. As outlined above, organic acids and APases secreted from plant roots are important responses to mobilize unavailable P in soils. It is hypothesized that the transport of organic acids is the key factor for the solubilization of sparingly soluble P in soil. In order to mobilize organic P in soil, mineralization is a key factor, although the mechanism of mineralization by APase is not still detailed. Therefore, it was focused on the roles of P mobilization by organic acids and APases secreted from plant roots and four experiments were conducted to prove the hypotheses.

In chapter 2, root exudates of *Arabidopsis thaliana* under P deficient condition were characterized. In chapters 3 and 4, malate and citrate transporters induced by P deficiency in Arabidopsis roots were characterized, respectively. In chapter 5, characteristics of the expression of genes for APases of *Lupinus albus* and mobilization of organic phosphate by exogenous phosphatase and phytase were investigated. The possibility of application for improvement of P utilizing efficiency by using genetic modification was also estimated. This approach will provide a basis for the development of sustainable agriculture.

Chapter 2

Characterization on phosphorus deficient tolerance of *Arabidopsis thaliana*

1. Introduction

As *Arabidopsis thaliana* is the most important model in the plant kingdom, the genome sequence was firstly completed (Arabidopsis Genome Initiative 2000). It allowed us to conduct comprehensive analyses of gene expressions and metabolic changes by the P deficiency. The transcriptomics, proteomic, and metabolomic analyses have been already carried out (e.g. Hammond et al. 2003; Tran et al. 2008; Thibaud et al. 2010). However, it is difficult to understand whole aspects of P mobilizing mechanisms by root exudates under P deficiency by the transcriptomic, proteomic, and metabolomic analyses targeted to intracellular samples. Therefore, this chapter aimed to elucidate the characteristics of exudates from roots of *Arabidopsis thaliana* grown under P deficient condition based on the metabolomics and in silico analyses.

2. Results

2-1. Plant growth and phosphorus status of *Arabidopsis thaliana*

Arabidopsis thaliana plants were aseptically cultivated in gel media containing half strength Murashige-Skoog basal salts with or without P. Fresh weight and P concentration of Arabidopsis shoots were measured, and total P content was determined (Fig. 2-1). Fresh weight and P content of plants grown under +P condition were significantly higher than –P condition at 10, 20 and 25 days after transferred (DAT) (Fig.2-1A and B). P concentration of plants grown

under +P condition was highest at 10 DAT. The P concentration of plants grown under –P condition was less than 2 mg-P/g DW at 20 and 25 DAT (Fig. 2-1C), indicating that the P treatments influenced on the P status of Arabidopsis plants as expected.

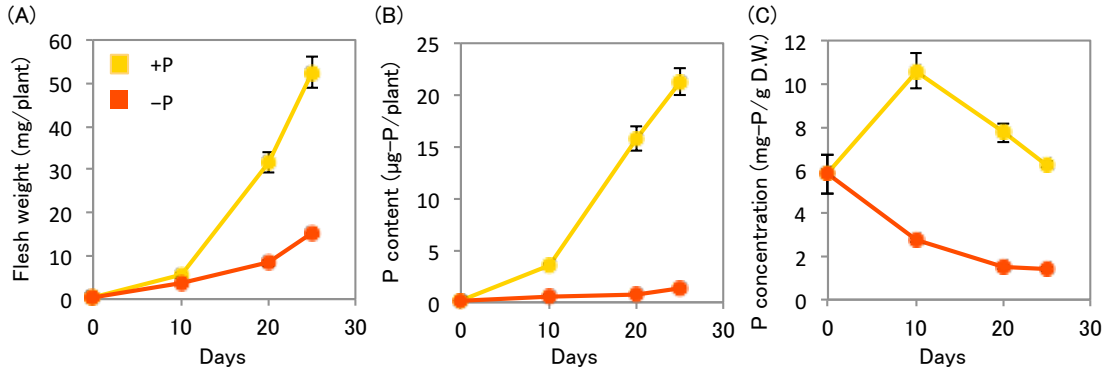

Figure 2-1 Flesh weight (A) and P content (B) and P concentration (C) of Arabidopsis shoot grown under P sufficient condition and P deficient condition.

2-2. Profiling of the detected metabolites in root exudates

To analyze comprehensive metabolic alterations of root exudates of Arabidopsis, metabolomics approach was conducted using GC/MS. The fresh weight of the roots at 10 DAT could not be measured because of the insufficient growth. Therefore, the root exudates of +P and –P plant roots were collected at 20 DAT. The result of metabolite profiling of the root exudates is shown in Table 2-1. As a result of an analysis of the GC/MS peaks, peaks for 39 compounds were detected in one GC/MS scan. There were 17 amino acid peaks and 9 organic acid peaks detected (Table 2-1). Among the organic acids, glyceric acid, pipecolic acid, citric acid, threonic acid, malic acids and fumalic acid were significantly induced by P deficiency. Among the sugars, sucrose of -P condition was increased 30.39 times compared to the +P condition. Moreover, galactose and arabinose was significantly induced by P deficiency. In case of amino acids, arginine, glutamine and threonine in the exudates were significantly induced by P deficiency.

Table 2-1 List of the compounds detected by GC/MS analysis. Data are presented as the mean ± standard error (*n*=3). Values followed by asterisks are significantly different from the +P condition according to Student's t-test results (**P*<0.05, ***P*<0.01). Red or blue characters indicate significant increased or decreased.

	Name	+P			−P			T−test
Amino acids	Arg	1.00	±	0.27	12.51	±	5.01	*
	Gln	1.00	±	0.17	5.70	±	2.59	
	Pro	1.00	±	0.29	4.07	±	1.87	
	Thr	1.00	±	0.19	3.87	±	1.09	*
	Lys	1.00	±	0.31	3.69	±	2.25	
	Asn	1.00	±	0.24	3.35	±	1.51	
	Glu	1.00	±	0.18	2.35	±	0.61	*
	Asp	1.00	±	0.18	2.06	±	0.48	
	Ala	1.00	±	0.33	2.01	±	1.04	
	Val	1.00	±	0.22	1.72	±	0.64	
	Tyr	1.00	±	0.22	1.71	±	0.62	
	Ile	1.00	±	0.22	1.63	±	0.51	
	Phe	1.00	±	0.19	1.61	±	0.73	
	β Ala	1.00	±	0.32	1.61	±	0.28	
	GABA	1.00	±	0.19	1.60	±	0.42	
	Gly	1.00	±	0.21	1.48	±	0.47	
	Ser	1.00	±	0.26	0.90	±	0.29	
Organic acids	Glyceric acid	1.00	±	0.17	6.46	±	0.46	**
	Pipecolic acid	1.00	±	0.48	5.01	±	1.70	*
	Citric acid	1.00	±	0.21	4.08	±	0.64	**
	Threonic acid	1.00	±	0.21	3.78	±	0.25	*
	Malic acid	1.00	±	0.19	3.54	±	0.17	**
	Fumaric acid	1.00	±	0.25	1.86	±	0.29	*
	Gluconic acid	1.00	±	0.15	1.36	±	0.09	
	Succinic acid	1.00	±	0.11	1.27	±	0.33	
	Lactic acid	1.00	±	1.09	0.26	±	1.47	
Sugars and Sugar alcohols	Sucrose	1.00	±	0.37	30.39	±	11.92	*
	Glucose	1.00	±	0.27	7.30	±	3.12	
	Galactose	1.00	±	0.15	3.75	±	0.29	**
	Glycerol	1.00	±	0.07	3.60	±	0.50	**
	Fructose	1.00	±	0.26	2.90	±	1.41	
	Fructose	1.00	±	0.28	2.76	±	1.21	
	Arabinose	1.00	±	0.10	2.05	±	0.21	**
	Ribose	1.00	±	0.15	1.12	±	0.27	
	myo−Inositol	1.00	±	0.11	0.89	±	0.12	
Others	Pyroglutamic acid	1.00	±	0.23	3.89	±	1.72	
	Suberyl	1.00	±	0.21	2.67	±	0.45	*
	Guanine	1.00	±	0.27	1.73	±	0.90	
	Phosphoric acid	1.00	±	0.02	0.21	±	0.03	**

2-3. Determination of organic acids content and APase activity in the exudates

Exudation of organic acids and APase is the key component for mobilization of unavailable P in soils. Thus, the substantial amount of organic acids and the activity of APase in the exudate of Arabidopsis roots were analyzed here.

The major forms of root-secreted organic acids under -P conditions are citrate and malate in the most plants (Jones 1989), therefore, citrate and malate contents in the exudates of Arabidopsis were quantitatively determined using the enzyme assay kits. The exudation of both malate and citrate from -P plants was significantly higher than +P plants (Fig. 2-2). However, the amount of malate and citrate in the root exudates of Arabidopsis was not high level compared to P-tolerant plants such as lupin (e.g. citrate; 1.16 μmol h^{-1} g root FW^{-1} in -P mature cluster roots, malate; 0.61 μmol h^{-1} g root FW^{-1} in -P normal roots, Neumann et al. 1999).

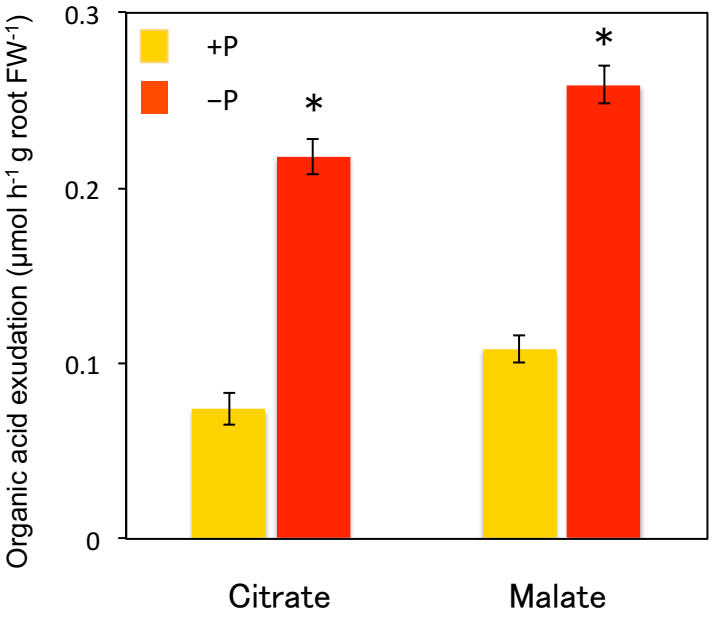

Figure 2-2 Malate and citrate exudation from *Arabidopsis thaliana* roots grown under P sufficient condition and P deficient condition. Data are presented as the mean ± standard error (*n*=3). Values followed by asterisks are significantly different from the +P condition according to Student's t-test test results (*P<0.05).

APase activity of the root exudate was determined by using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate. Similar to other plants, APase from Arabidopsis roots under P deficient condition was higher than P sufficient condition (Fig. 2-3). The level of APase activity under P deficiency was similar level to the other crop plants analyzed in previous study (Tadano and Sakai 1991), although the increasing rate in P deficiency was not very high in Arabidopsis (Fig. 2-3).

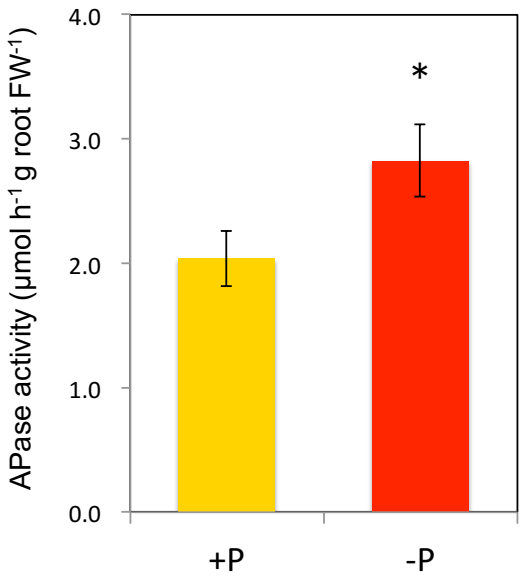

Figure 2-3 APase activity of root exudates of Arabidopsis grown under P sufficient and P deficient condition. Data are presented as the mean ± standard error (*n*=24). Values followed by asterisks are significantly different from the +P condition according to Student's t-test test results (*P<0.05).

2-4. Transcriptomic analysis by microarray

Purple acid phosphatase (PAP) family proteins are the major phosphatase in plants. It was found that 29 PAP family proteins existed in Arabidopsis genome (Li et al. 2002). Previously, Choi (2009) conducted transcritomic analysis of P deficient Arabidopsis root and shoot by microarray. As the result of in silico analysis of the data, it was revealed that various genes were induced or repressed by P deficiency. The expression data of PAPs was extracted from the

transcriptomic analysis. Among 29 genes for APase in Arabidopsis, *AtPAP6, 7, 10, 12, 17, 22, 23, 24*, and *25* were up-regulated by P deficient condition in root, on the other hands, AtPAP3 was down-regulated (Table 2-2). This was corresponded to the data by Tran et al. (2010), who reported that AtPAP12 and AtPAP26 are the predominant PAP isozymes secreted by P deficient Arabidopsis root. Recently, Wang et al. (2011) concluded AtPAP10 was the APase associated with the root surface after secretion.

Table2-2 List of the gene expression of AtPAPs in the root and shoot of Arabidopsis detected by microarray analysis. Fold change (FC) means signal Intensity (SI) of –P/+P condition. Experiment 1 and experiment 2 indicate replications. Red characters indicate more than double.

		Root						Shoot					
		Experiment1			Experiment2			Experiment1			Experiment2		
			SI			SI			SI			SI	
AGI code	Name	FC	+P	-P	FC	+P	-P	FC	+P	-P	FC	+P	-P
AT1G14700	AtPAP3	0.59	406	240	0.63	523	328	0.66	3524	2313	0.50	4275	2153
AT1G15080	AtPAP2	0.76	317	241	0.94	249	235	0.77	249	191	1.07	205	219
AT1G52940	AtPAP5	1.02	125	128	0.72	133	97	1.32	110	145	0.96	161	154
AT1G56360	AtPAP6	10.88	19	209	9.89	30	293	2.10	27	58	2.11	31	66
AT2G01180	AtPAP1	0.86	2228	1926	1.21	1858	2244	3.04	4969	15092	1.63	2992	4889
AT2G01880	AtPAP7	3.99	2118	8452	3.35	1849	6197	1.02	455	463	0.92	532	487
AT2G01890	AtPAP8	1.05	5241	5480	0.95	6610	6301	0.99	2193	2209	1.11	2034	2250
AT2G03450	AtPAP9	1.25	4541	5676	1.28	3955	5045	0.98	2078	2029	1.03	2011	2069
AT2G16430	AtPAP10	5.70	1205	6869	5.37	1166	6258	1.88	3619	6813	1.48	4099	6056
AT2G18130	AtPAP11	1.09	852	931	1.01	1201	1193	1.15	927	1062	0.90	947	856
AT2G27190	AtPAP12	6.51	3389	22061	5.69	3306	18823	4.45	2605	11594	4.60	1633	7518
AT2G32770	AtPAP13	1.04	422	439	1.25	448	562	0.89	580	517	0.82	583	476
AT2G46880	AtPAP14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AT3G07130	AtPAP15	1.62	816	1319	1.19	862	1028	1.13	394	447	0.97	433	418
AT3G10150	AtPAP16	1.54	88	136	1.46	122	177	4.84	132	638	4.29	155	667
AT3G17790	AtPAP17	36.33	1395	50669	43.57	847	36901	30.43	1105	33632	35.09	890	31216
AT3G20500	AtPAP18	1.05	1246	1306	0.96	1042	1001	1.51	1390	2100	1.44	1440	2072
AT3G46120	AtPAP19	0.98	55	54	0.79	134	106	0.91	89	81	1.52	55	83
AT3G52780	AtPAP20	0.86	224	192	0.93	311	288	1.04	316	330	1.06	283	298
AT3G52810	AtPAP21	1.00	21	24	1.00	25	21	1.00	26	29	3.42	33	112
AT3G52820	AtPAP22	6.22	588	3661	6.02	534	3216	1.71	270	460	2.17	174	379
AT4G13700	AtPAP23	10.47	111	1164	7.07	115	811	1.07	232	247	1.50	117	175
AT4G24890	AtPAP24	2.05	726	1486	1.78	833	1484	3.00	630	1890	4.36	516	2250
AT4G36350	AtPAP25	7.78	18	143	4.13	27	112	6.07	19	113	1.48	145	214
AT5G34850	AtPAP26	1.21	8011	9732	1.15	7865	9019	1.02	10672	10904	1.06	11823	12515
AT5G50400	AtPAP27	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AT5G57140	AtPAP28	1.69	77	130	0.84	106	89	0.94	81	76	0.71	110	79
AT5G63140	AtPAP29	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
negative control		1.10	20	25	1.11	28	20	1.10	21	33	1.16	29	19

The three homologs of PAPs were also isolated from white lupin and designated LASAP1, LASAP2 and LASAP3 (Wasaki et al. 1999, 2000, 2003). Phylogenetic tree of all Arabidopsis PAPs and three isolated PAPs of white lupin was prepared to estimate the structural similarities among PAPs (Fig. 2-4). AtPAP10, AtPAP12 and AtPAP26 belonged to a clade containing LASAP1 and LASAP2, which had been reported as the non-specific APases (Wasaki et al. 1999, 2000). It is suggested that PAPs in the clade are the major players in mobilization of organic P in the rhizosphere.

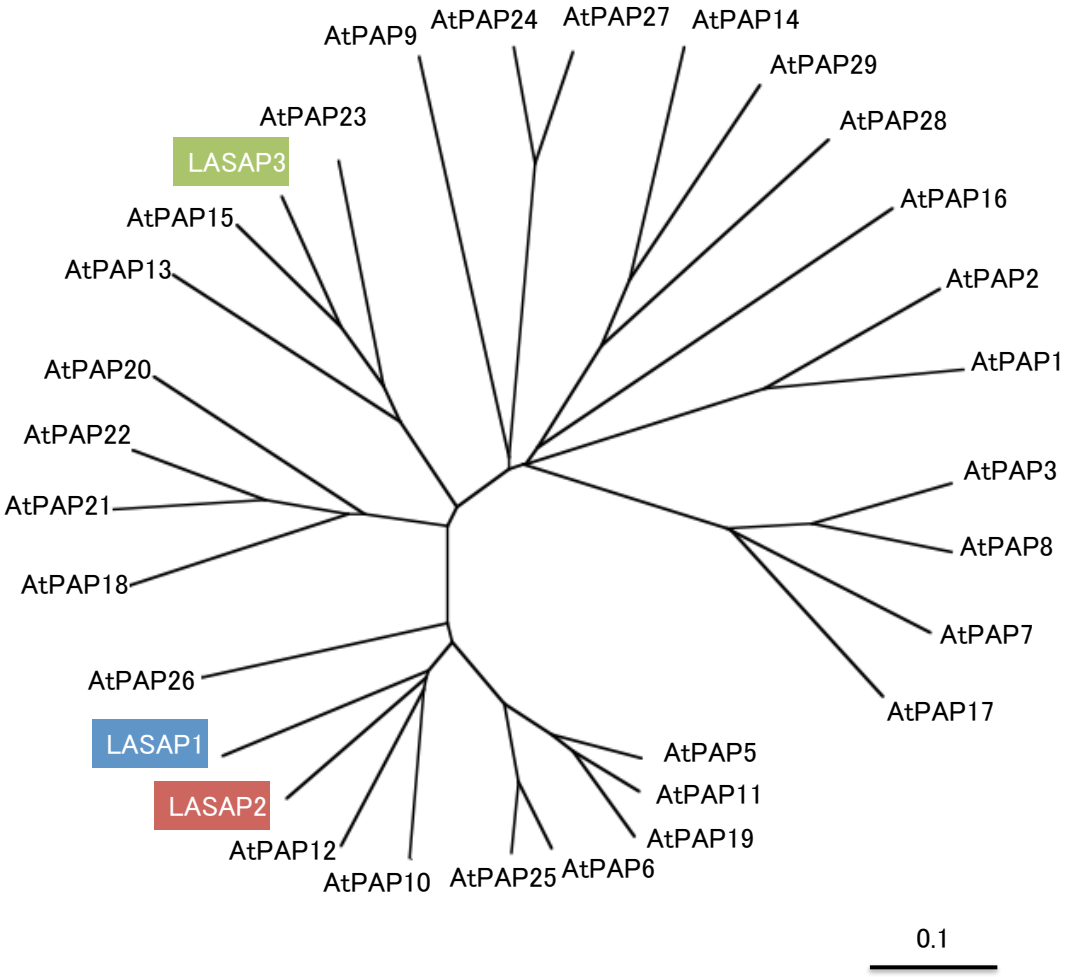

Figure 2-4 Phylogenetic relationship of AtPAP proteins in *Arabidopsis thaliana* and LASAP1, 2 and 3 in *Lupinus albus*. The deduced amino acid sequences were aligned by ClustalW.

3. Discussion

3-1. Characteristics of low molecular weight compounds in root exudates of Arabidopsis grown under P deficient conditions

Root exudates contain sugars, amino acids, organic acids, flavonoids, enzymes, and nucleotides (Rovira 1969). In P deficient conditions, it has been known that plants secrete various metabolites, such as organic acids, strigolactones and flavonoids (Gardner et al. 1983; Weisskopf et al. 2006). Recently, it was enabled to analyze comprehensive metabolites in the organisms, so-called 'metabolomics', by using mass spectrometer. The novel concept 'secretome', i.e. the comprehensive analysis of root exudate was also proposed to understand the nutrient dynamics or plant-microbial interactions. Thus, it was analyzed that the secretome components in root exudates of Arabidopsis was analyzed by GC/MS, although this method could not give quantitative values. It was revealed in this study that secretion of many organic acids, including malate and citrate, amino acids and sugars increased in the exudates of P deficient Arabidopsis roots (Table 2-1). The data suggested that the secretomic approach could be a valuable tool for characterization of complex responses for nutrient stresses in the future.

Increase of arginine, glutamine and threonine in the exudates by P deficiency were suggested (Table 2-1). Tawaraya et al. (in press) were also analyzed the root exudate of rice by secretomic approaches using CE-TOF MS, and they found that several amino acids in root exudates were altered by P status. It has been reported that some amino acids, such as arginine, serine and cysteine, are affect the growth of rhizosphere microorganisms (Griffiths et al. 1999). The increased exudation of amino acids may contribute to the modification of the microbial community structures of the rhizosphere including the phosphate-solubilizing bacteria. Interestingly, sucrose in the root exudates of –P samples was induced about 30 times higher than in +P condition (Table 2-2). Under P starvation, plants modify sugar metabolisms and accumulate starch in

their leaves. Increased loading of sucrose to the phloem under P starvation not only functions to relocate carbon resources to the roots, which increases their size relative to the shoot, but also has the potential to initiate sugar-signaling cascades. The sugar-signaling alter the expression of genes involved in optimizing root biochemistry to acquire soil P through increased expression and activity of inorganic phosphate transporters, the secretion of APases and organic acids to release P from the rhizosphere, and the optimization of internal P use (Hammond and White 2008, 2011). Moreover, Zhou et al. (2008) reported that exogenous sucrose in media was influenced to the formation of cluster roots of white lupin under P deficiency. Therefore, sucrose from root to the rhizosphere might have the role for the signaling molecule of the modification of root architectures.

Predictably, root-secreted malate and citrate were also induced by P deficiency in quantitative evaluation (Table 2-1, Fig. 2-2). Increased root exudation of citric acid, malic acid and oxalic acid under P deficient condition has been reported in *Lupinus albus* (Gardner et al. 1983), *Brassica napus* (Hoffland et al. 1989), and *Cicer arietinum* (Ohwaki and Hirata 1992). It was also reported that many organic acids in root exudates of rice were altered by P status (Tawaraya et al. in press). It was shown that malate exudation was not altered by P deficiency in rice, and citrate in the exudate significantly decreased. It can be concluded that major contributors among organic acid molecules to P mobilization are diverse among plant species.

The responses of minor organic acids were also suggested in this study (Table 2-1). An increase of glyceric acid in Arabidopsis exudates by P deficiency was found, although it was contradicted in rice (Tawaraya et al. in press). Increases of pipecolic acid and threonic acid in root exudates of P deficient condition found in this study (Table 2-1) have not been reported yet. Further studies are required to understand the function of minor organic acids responded to P status shown in this study.

3-2. Characteristics of APase, citrate and malate in root exudates of Arabidopsis grown under P deficient conditions

Activity of root-secreted APase in Arabidopsis was significantly high in P deficient conditions than in P sufficient conditions (Fig. 2-3). It was suggested by microarray analysis that many APase homologs were induced by -P (Table 2-2). These results are consistent with previous reports. Tran et al. (2010) reported that AtPAP12 and AtPAP26 are the predominant PAP isozymes secreted by P deficient Arabidopsis root. Moreover, Hurley et al. (2010) reported that AtPAP26 is the principal contributor to P deficiency inducible APase activity, and that it plays an important role in the Pi metabolism of P deficient Arabidopsis. In addition, Wang et al. (2011) investigated and concluded that AtPAP10 was induced by P limitation at both transcriptional and posttranscriptional levels and associated with the root surface after secretion. They suggested that AtPAP10 played an important role in plant tolerance to P deficiency.

Gene expression of *AtPAP15* was not so much induced by -P (Table 2-2). It has been reported that AtPAP15 has phytase activity and hydrolyzes phytate to myo-inositol and free Pi (Zhang et al. 2008; Kuang et al. 2009). They mentioned that AtPAP15 might be not secreted from root under P deficient condition, although the mRNA accumulation was in the root. AtPAP15 was the closest homolog among AtPAPs to LASAP3 (Fig. 2-4), which is the white lupin PAP homolog with a phytate-specific activity (Kaneko 2004). Similarly, it is not clarified whether LASAP3 is secreted protein or not, therefore, further investigation on the expression properties of LASAP3 is required to understand the functions of these phytases.

In conclusion, Arabidopsis also have several mechanisms responded to P-deficiency as found in other plants in general. However, the amount of malate and citrate in the root exudates was not high level such as P-tolerant plants such as white lupin. APase activity secreted from Arabidopsis roots under P-deficient conditions was also low level rather than white lupin as indicated by Tadano and

Sakai (1991). These facts suggest that the function of P mobilization in Arabidopsis is not very high.

4. Materials and methods

4-1. Growth condition and collection of root exudates of *Arabidopsis thaliana*

Surface-sterilized seeds of *Arabidopsis thaliana* (ecotype Columbia; Col-0) were germinated in 10×10 cm plastic dishes containing sterile Murashige–Skoog basal salts at half strength and 0.5% gellan gum (pH 5.6; 1/2 MS media) for 4 days. Seedlings were transferred to same dish containing +P (0.625 mM) or –P (0 mM) 1/2 MS media. After 10, 20 and 25 days, cultivated plants were transferred to inclined square dishes containing 2 mL distilled water in a corner of the dish after washing with distilled water (Fig. 2-5). The root exudates were collected for three hours.

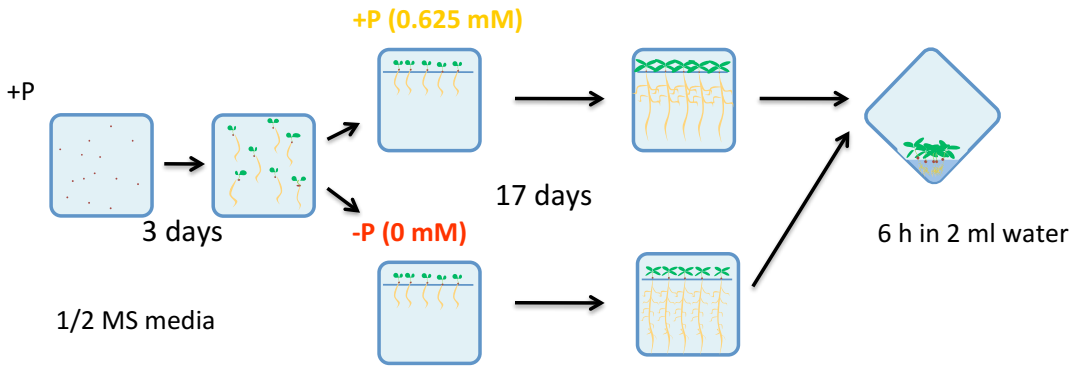

Figure 2-5 The system for growing and collection of root exudates of *Arabidopsis thaliana*.

4-2. Metabolomic analysis of root exudates of *Arabidopsis thaliana*

The collected root exudates were used for metabolomics analysis by GC/MS. The GC/MS analysis was kindly conducted by Keiki Okazaki in National Agricultural Research Center for Hokkaido Region. GC/MS peaks were identified by RI and mass spectrum based on Okazaki et al. (2008) and Suzuki et al. (2009). Citrate and malate contents in the exudates were determined using the enzyme assay kits (E-kit; Roche Diagnostics, Basel, Switzerland).

4-3. Measurement of APase activities

The collected root exudates were used for measurement of APase activity. APase activity of root exudate was determined by using *p*-NPP as a substrate according to the Gilbert et al. (1999).

Chapter 3

Characterization of malate transporter induced by phosphorus deficiency

1. Introduction

In *Arabidopsis thaliana*, *TaALMT1*-like genes form a small protein family of 14 members (Hoekenga et al. 2006). For example, AtALMT1 is malate transporter involved in Al tolerance (Hoekenga et al. 2006), AtALMT9 is vacuolar malate channel (Kovermann et al. 2007) and AtALMT12 is anion transporter involved in stomatal closure (Sasaki et al. 2010). These reports indicated that AtALMT family genes have multiple functions in *Arabidopsis thaliana*, although some of them were not well characterized yet, including the relationships in the malate secretion under P-deficient conditions. In this chapter, it was revealed that AtALMT3, a homolog of the AtALMT family genes, was involved in malate transport induced by P deficiency.

2. Results

2-1. Expression of *AtALMTs* under phosphorus deficient condition

First, the expression pattern of *AtALMT* genes in both shoots and roots of Arabidopsis thaliana grown under +P and -P conditions was compared by microarray analyses (Table 3-1). *AtALMT1* was accumulated only in roots and not induced by P deficiency. *AtALMT3* was also root specific and higher in -P than +P. Some of *AtALMTs*, e.g. *AtALMT6*, were shoot specific. *AtALMT9* transcripts were found mainly in roots but it was not regulated by P nutrient. *AtALMT12* seemed constitutive in whole plant both +P and -P conditions.

Table 3-1 List of the gene expression of *AtALMTs* in the root and shoot of Arabidopsis detected by microarray analysis. Fold change (FC) means signal Intensity (SI) of –P/+P condition. Experiment 1 and experiment 2 indicate replications. Red and blue characters indicate more than double or less than half.

		Root						Shoot					
		Experiment1			Experiment2			Experiment1			Experiment2		
			SI			SI			SI			SI	
Name	AGI	FC	+P	-P	FC	+P	-P	FC	+P	-P	FC	+P	-P
AtALMT1	At1g08430	0.20	587	115	0.33	444	148	1.14	121	138	1.12	126	140
AtALMT2	At1g08440	1.32	29	39	1.37	26	36	0.79	90	71	1.08	58	62
AtALMT3	At1g18420	2.45	265	649	2.79	281	784	0.97	59	58	0.52	53	28
AtALMT4	At1g25480	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtALMT5	At1g68600	0.51	42	21	1.31	59	78	2.68	1396	3743	2.97	1100	3272
AtALMT6	At2g17470	1.00	21	22	1.00	27	20	1.15	88	102	1.79	94	167
AtALMT7	At2g27240	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtALMT8	At3g11680	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtALMT9	At3g18440	1.06	1454	1546	1.28	1160	1481	1.02	1390	1420	1.26	1102	1387
AtALMT10	At4g00910	0.44	608	270	0.30	840	251	1.00	21	29	1.00	28	20
AtALMT11	At4g17585	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtALMT12	At4g17970	2.85	76	215	2.54	81	206	2.14	362	774	2.98	376	1123
AtALMT13	At5g46600	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtALMT14	At5g46610	0.93	156	145	1.17	132	155	0.77	148	114	1.02	147	149
negative control		1.10	20	25	1.11	28	20	1.10	21	33	1.16	29	19

Deduced amino acid sequences of AtALMTs were compared to estimate the similarity of protein structures (Fig. 3-1). AtALMTs can be classified three groups in the phylogenetic tree (Fig. 3-1). Amino acid sequence of AtALMT3 was the most similar to AtALMT9 with 56% identity. AtALMT3 and AtALMT1, a malate transporter induced by Al stress, were belonged to the different clades and showed only 24% identities (Fig. 3-1).

RT-PCR was performed to certify the results showed by microarray analyses (Fig. 3-2A). The data of RT-PCR showed similar trends with the results of microarray analyses. For the further confirmation, quantitative real-time RT-PCR (qRT-PCR) was performed for transcripts of *AtALMT1* and *AtALMT3*. qRT-PCR revealed that mRNA of *AtALMT3* was significantly up-regulated in -P than in +P condition, while that of AtALMT1 was down-regulated (Fig. 3-2B). AtIPS1, a P deficiency response non-coding RNA belonging to *TPSI1/Mt4* family, was also significantly up-regulated by -P condition (Fig. 3-2B).

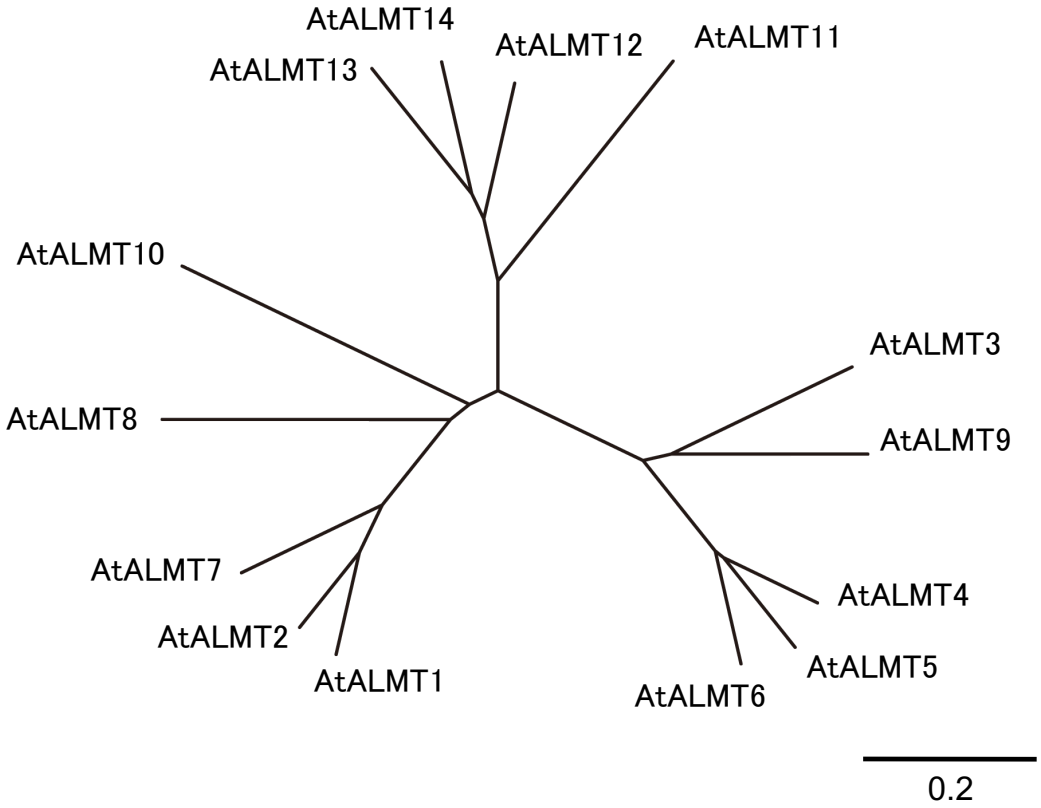

Figure 3-1 Phylogenetic relationship of AtALMT proteins in *Arabidopsis thaliana*. AtALMT3 was 24% identical to AtALMT1. The deduced amino acid sequences were aligned by ClustalW.

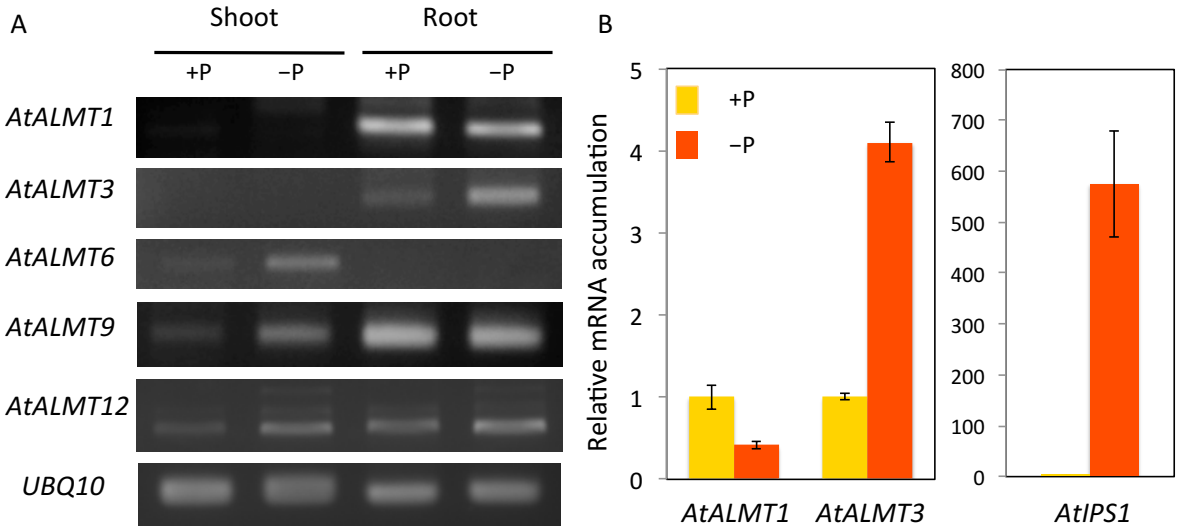

Figure 3-2 mRNA accumulation of *AtALMTs, UBQ1 and AtIPS1* analyzed by semi-quantitative RT-PCR, A, and quantitative RT-PCR ,B. qRT-PCR was performed for cDNAs of *Arabidopsis thaliana* roots cultivated under phosphorus sufficient condition and phosphorus deficient condition with triplicate (error bar = SE). *AtIPS1* is a P starvation responsive non-coding RNA belonging to *TPSI1/Mt4* family.

2-2. Loss-of-function analysis

To analyze the function of *AtALMT3*, we obtained two *AtALMT3* mutant lines (*atalmt3-1* and *atalmt3-2*, provided by Salk institute as Salk_013699 and CS841593, respectively). T-DNA was inserted at 6 bp upstream of the stop codon and 248 bp upstream of the start codon in *atalmt3-1* and *atalmt3-2*, respectively (Fig. 3-3).

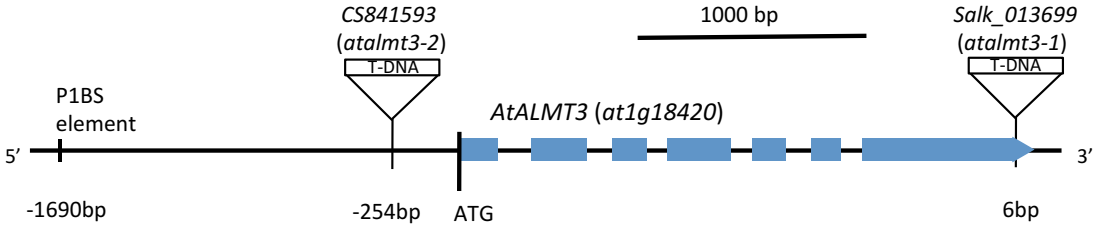

Figure 3-3 Schematic of the *AtALMT3* locus (*at1g18420*) showing T-DNA insertion sites and P1BS element site.

The mRNA was significantly down- and up-regulated compared to WT in *atalmt3-1* and *atalmt3-2*, respectively (Table 3-2, Fig. 3-4A). It is considered that T-DNA sequence might act as enhancer in *AtALMT3* expression in *atalmt3-2* mutant.

Table 3-2 *AtALMT1* and *AtALMT3* gene expressions of WT, *atalmt3-1*, *atalmt3-2* and *atalmt1*. qRT-PCR was performed for cDNAs of *Arabidopsis thaliana* roots cultivated under phosphorus sufficient condition and phosphorus deficient condition with triplicate.

	WT		*atalmt3-1*		*atalmt3-2*		*atalmt1*	
	+P	-P	+P	-P	+P	-P	+P	-P
AtALMT3	1.00±0.20	1.69±0.12	0.45±0.09	0.74±0.04	70.39±12.19	17.78±1.23	0.70±0.12	0.91±0.31
AtALMT1	1.00±0.06	0.54±0.01	1.03±0.11	0.75±0.07	0.67±0.12	0.58±0.07	n.d.	n.d.

The exudation of both malate and citrate from -P plants was higher than +P plants in all lines (Fig. 3-4B). Malate exudation of *atalmt3-1* decreased by 34% compared to WT in –P condition, although it was not significant (P=0.08). On the

other hand, that of *atalmt3-2* increased by 31% (P=0.05). There were no differences of citrate exudation among three lines (Fig. 3-4B). These results suggested that AtALMT3 was involved in the malate exudation from Arabidopsis roots induced by P-deficiency.

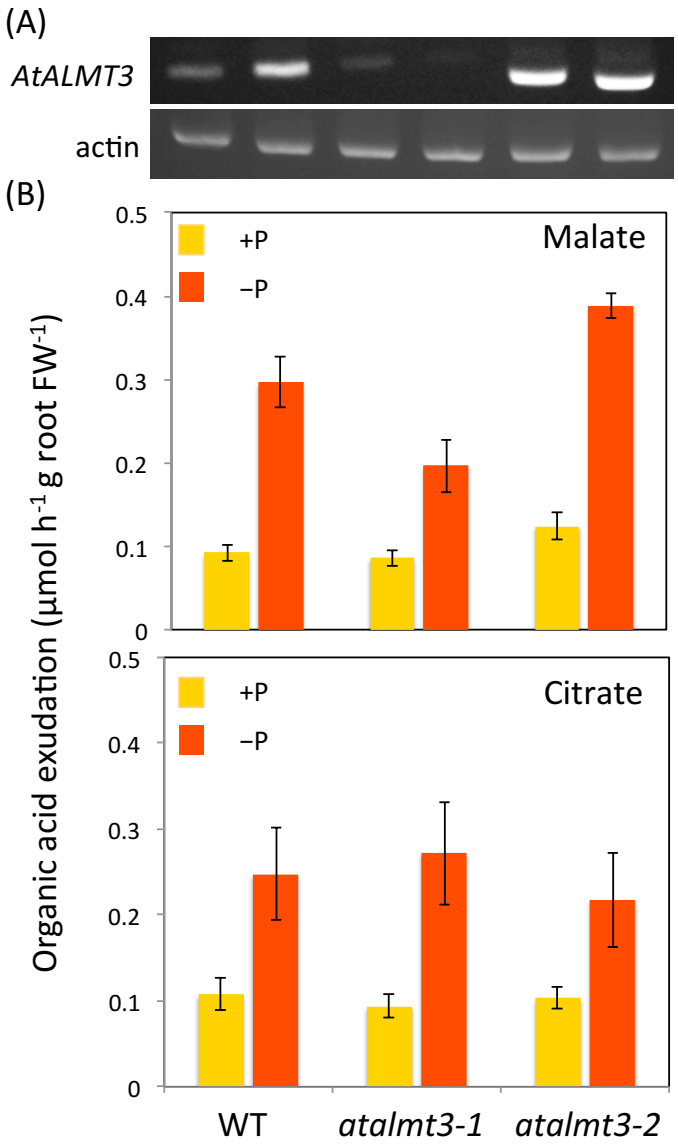

Figure 3-4 Effect of P nutrition on mRNA accumulation (A) and malate and citrate exudation from *Arabidopsis thaliana* roots (B). (*n* = 3, error bar = SE)

2-3. Histochemical and subcellular localization of *AtALMT3*

To investigate histochemical localization of *AtALMT3* expression, I have generated transgenic *Arabidopsis thaliana* plants harboring a reporter gene, ß-glucuronidase (GUS), under the control of the putative *AtALMT3* promoter (2,049 bp upstream from the start codon). *AtALMT3* promoter::GUS lines showed strong expression in epidermal cells and root hairs, especially in the adjacent region of the meristem of roots (Fig. 3-5A-F). There were no differences of the localization of GUS activities between +P and -P condition (Fig. 3-5C,D). *AtALMT3* expression was not detected in shoot tissues (Fig. 3-5G).

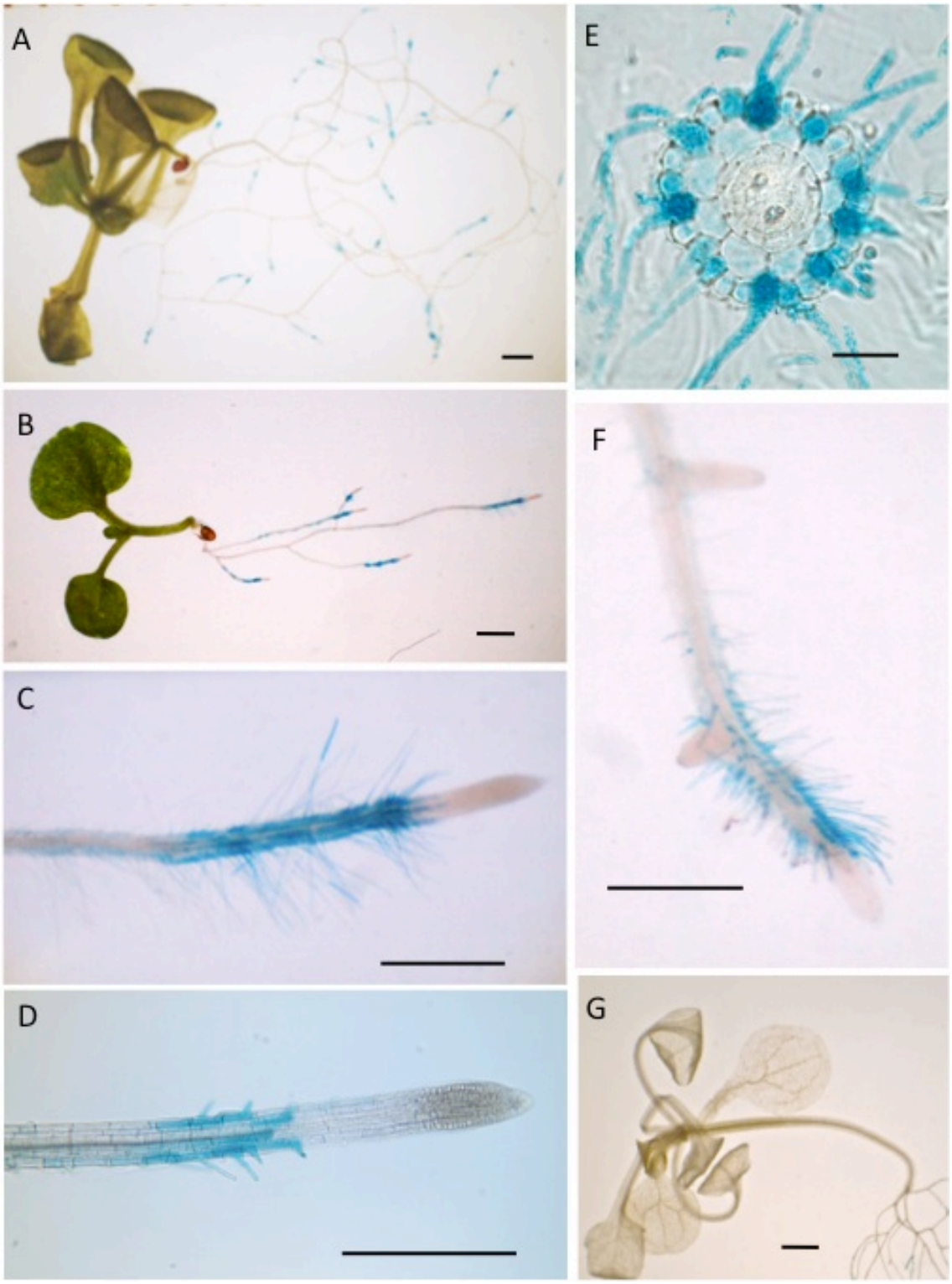

Figure 3-5 Localization of *AtALMT3* promoter::GUS in transgenic seedlings. Transgenic plants carrying a *AtALMT3* promoter::GUS construct were used for GUS staining after cultivated +P condition for 8 day (D) and –P condition for 20 Days, (A, E and G), and 8 Days, (B, C and F). A and B, Whole plant. C and D, Region behind the root tip. E, Cross-sectional view of root. F, Portion of root tip and meristematic regions of lateral roots. G, Shoot. Bar=1mm (A, B and G), 500 μm (C, D and F) and 50 μm (E).

To investigate subcellular localization of AtALMT3, AtALMT3::GFP fusion protein was transformed into Arabidopsis under the control of NP (native promoter). Plasma membranes were stained by a short incubation with the hydrophobic red fluorescent dye FM4-64 (Fig. 3-6F), which was found to colocalize with the green AtALMT3-dependent fluorescence after the two separate images were merged (Fig. 3-6G). It was revealed that NP::AtALMT3::GFP protein located at root hair cells and located in plasma membrane (Fig. 3-6).

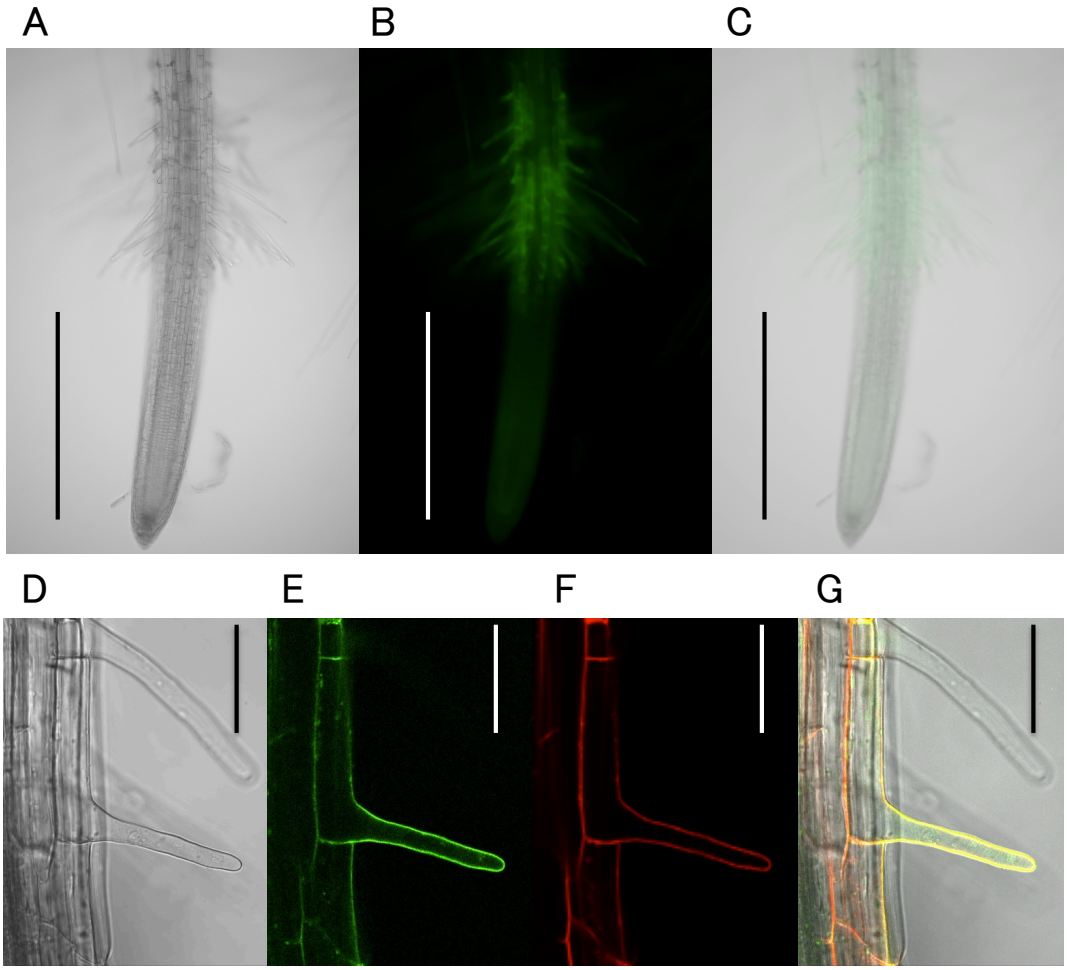

Figure 3-6 Localization of *AtALMT3* promoter::AtALMT3::GFP protein in transgenic seedlings. Transgenic plants were grown under P deficient condition for 10 days were used. Bright field image (A and D), fluorescence image (B and E), plasma membrane were stained red with FM4-64 (F), and merged image (C and G). Bar=500 μm (A, B and C) and 50 μm (D, E, F and G).

2-4. Growth of the AtALMT1 and AtALMT3 mutants in soil culture

To investigate the role of AtALMT3, the mutants and wild type plants were cultivated in the soil. The growth and P content of shoot were shown in Table 3-3. The differences of both growth and P content were not significantly among plant lines, although P treatments significantly influenced to their growth and P accumulation. The relationships between the growth and P content of each pot were shown in Fig. 3-7. Interestingly, P uptake ability of *atalmt3-1* and *atalmt1* was lower compared to WT, while *atalmt3-2* was similar to WT (Fig. 3-7). Reduction rate of P uptake in *atalmt1* was bigger than *atalmt3-1* (Fig. 3-7).

Table 3-2 *AtALMT1* and *AtALMT3* gene expressions of WT, *atalmt3-1*, *atalmt3-2* and *atalmt1*. qRT-PCR was performed for cDNAs of *Arabidopsis thaliana* roots cultivated under phosphorus sufficient condition and phosphorus deficient condition with triplicate.

	P level (mg-P kg-soil^{-1})											
	0				100				300			
(a) Dry weight (mg/plant)												
WT	0.81	±	0.08	a	4.06	±	0.47	abcde	5.05	±	0.46	bcdef
atalmt3-1	1.78	±	0.14	abc	5.46	±	0.11	cdef	7.20	±	1.10	ef
atalmt3-2	0.52	±	0.07	a	2.86	±	0.63	abcd	5.31	±	1.29	cdef
atalmt1	1.05	±	0.36	ab	6.11	±	1.08	def	9.15	±	1.55	f
(b) P content (ug-P/plant)												
WT	2.33	±	0.26	A	12.32	±	0.61	ABCD	20.88	±	1.97	CD
atalmt3-1	3.80	±	0.47	A	11.45	±	0.98	ABCD	22.82	±	5.31	D
atalmt3-2	1.37	±	0.20	A	6.74	±	1.31	AB	17.82	±	4.54	BCD
atalmt1	1.24	±	0.49	A	9.68	±	1.41	ABC	18.14	±	3.47	BCD

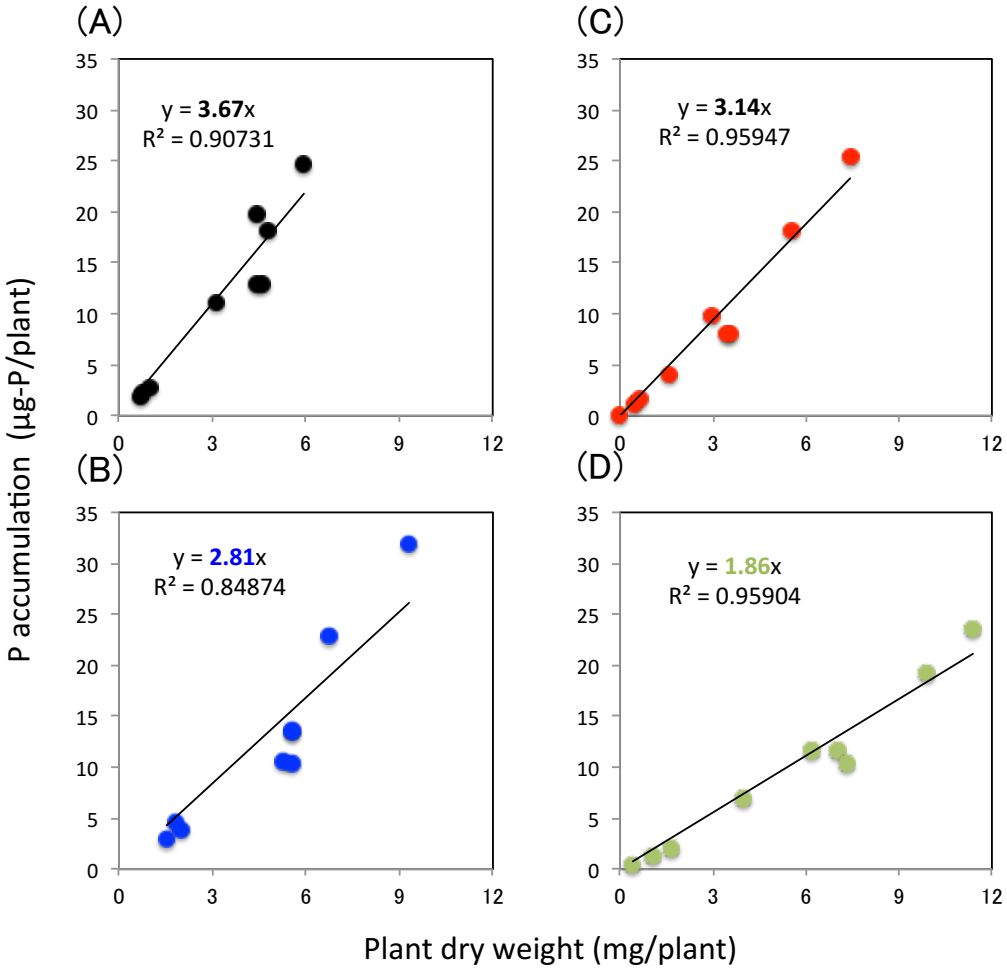

Figure 3-7 Relationship between plant dry weight and P accumulation of shoots of WT (A), *atalmt3-1* (B), *atalmt3-2* (C) and *atalmt1* (D).

3. Discussion

3-1. AtALMT3 is involved in malate transport under phosphorus deficiency in *Arabidopsis thaliana*

Under phosphorus deficient condition, plants secrete organic anions such as malate and citrate into the rhizosphere to mobilize insoluble P, which is bound with the metal ions such as Ca, Fe and Al (Raghothama 1999; Vance et al. 2003). It is considered that the organic acid transporters play very important roles on utilization of insoluble P in soils, although the P-deficiency responsive organic acid transporter genes have not yet isolated.

AtALMT3 gene expression was only found in roots and up-regulated by P-deficiency (Fig. 3-2). It was predicted that AtALMT3 had 6 transmembrane domains (data not shown). AtALMT3 showed highest similarity to AtALMT9, which was reported localized in the vacuolar membrane. *AtALMT9* was not up-regulated by P-deficiency in roots (Fig. 3-2A). While, gene expression of *AtALMT1*, the aluminum activated malate transporter localized in roots, was down-regulated (Fig. 3-2B). It has been suggested that the mechanisms of organic acid secretion are different between P-deficiency and Al stress (Ma 2005). Therefore, *AtALMT3* can be targeted as a candidate gene for malate transporter induced in roots of P-deficient *Arabidopsis thaliana*.

In fact, malate exudation under P deficiency reduced in the *AtALMT3* knockdown mutant, *atalmt3-1*, and increased in overexpression mutant, *atalmt3-2* (Fig. 3-4). This indicates that AtALMT3 is involved in malate exudation from Arabidopsis roots under P deficient conditions. However, the malate transport activity could not be detected by several electrophysiological analyses of AtALMT3 using oocytes of *Xenopus laevis* (data not shown). Kobayashi et al. (2007) reported that malate release from AtALMT1 was regulated both transcriptional and posttranscriptional levels. AtALMT3 might have also posttranscriptional regulation. It might be reason why 10.5 times up-regulation of AtALMT3 caused only 1.3 times

higher malate exudation in *atalmt3-2* (Fig. 3-4). In addition, Fetter et al. (2004) reported that heteromerization of maize (*Zea mays*) plasma membrane aquaporins ZmPIP1 and ZmPIP2 were required to act as a functional water channels. Therefore, AtALMT3 might have the interaction of other protein such as other ALMT family protein.

P uptake ability of the mutants and WT was compared by soil culture. pH of soil used in this experiment was 5.45, therefore, the Al stress was not critical. P uptake efficiency of *atalmt3-1* and *atalmt1* from soil was less than WT (Fig. 3-7). Thus, it is concluded that not only AtALMT3 but also AtALMT1 is involved in P mobilization in the rhizosphere.

3-2. Localization of AtALMT3 is linked to other functions for P uptake from the rhizosphere in *Arabidopsis thaliana*

Skene (2003) reported that exudation of low molecular weight exudates and phosphate uptake could be seen to peak at a region just behind the root tip. From results of promoter analyses, it is clearly demonstrated that AtALMT3 is specifically expressed in epidermal cells and root hairs, especially in the adjacent region of the meristem of roots, just behind the root tip (Figs. 3-5 and 3-6). NP::AtALMT3::GFP analysis also demonstrated that AtALMT3 located in plasma membrane of root hair cells (Fig. 3-6). These results suggest that AtALMT3 transport malate from inside to outside of epidermal and root hair cells under P-deficient condition. Root hair is very important to uptake nutrient, especially P, from soil (Gahoonia et al. 1998). In addition, the phosphate transporter genes of Arabidopsis (*Pht1;2*) or barley (*HvPht1;1*) also up regulated in root hair under P-deficient condition (Karthikeyan et al. 2002; Mudge et al. 2002; Schunmann et al. 2004). Furthermore, in our previously study, acid phosphatase activity was high in the root hair cells of hydroponically and soil-cultured white lupin under P-deficient conditions (Wasaki et al. 2008). Thus, plant has a very efficient system to secrete organic acids and phosphatases and uptake P by the phosphate transporter in

same tissues.

In conclusion, results obtained in this chapter suggest that AtALMT3 has properties that allow it to function as a malate efflux transporter localized plasma membrane of epidermis and root hair cells induced by P-deficiency, although the activity of malate transport was still not directly detected.

4. Materials and Methods

4-1. Arabidopsis accessions

Arabidopsis thaliana (Col-0) was used for all of the control experiments. The T-DNA insertion mutants of AtALMT3, *atalmt3-1* (Salk_013699), *atalmt3-2* (CS841593) and AtALMT1 knockout mutant, *atalmt1* (Salk_009629) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous plants were identified by PCR using appropriate primer sets (Table 3-4).

4-2. Plant material and growth condition

Surface-sterilized seeds of each Arabidopsis line were germinated in 10 x 10 cm plastic dishes containing sterile 1/2 media and 0.5% gellan gum (pH 5.6) for 4 days. Seedlings were transferred to same sized dishes containing 1/2 MS media with or without 0.625 mM phosphate. After 20 days, cultivated plants were transferred to inclined square dishes containing 2 mL distilled water in a corner of the dishes after washing with distilled water. The root exudates were collected for 6 h. Citrate and malate contents in the exudates were determined using the enzyme assay kits (E-kit; Roche Diagnostics). Fresh weight of root and shoot were weighted and immediately frozen in liquid N_2, and kept at -80˚C until use for RNA isolation.

4-3. RT-PCR and quantitative RT-PCR

Total RNA was extracted using the Agilent Plant RNA Isolation Mini kit (Agilent Technologies, Santa Clara, IL, USA) as recommended by the manufacturer. Total RNA (300 ng) was reverse-transcribed into cDNA in a 10 μL reaction using the SuperScript® VILO™ cDNA Synthesis Kit (Invitorogen, Carlsbad, CA, USA). The cDNAs and specific primer sets (Table 3-4) were used for RT-PCR and qRT-PCR. RT-PCR was carried out 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min using Ex taq (Takara-bio, Otsu, Japan). PCR products were separated by 2% agarose gel and stained with SYBR Green I, detected with Lumivision (Aisin, Kariya, Japan). Quantitative real-time RT-PCR was performed using an ABI StepOne real-time PCR system (Applied Biosystems, Piscataway, CA, USA) and SYBR Green kit (Takara-bio). The real-time PCR process comprised an initial denature at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, and a final dissociation stage of 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The data were analyzed using the ABI StepOne system software (Applied Biosystems). Transcripts were relatively quantified using actin transcripts as a control. Primer sequences for using RT-PCR and qRT-PCR are shown in Table 3-4.

Table 3-4 List of primers used in this chapter.

Target gene	Primer name	Sequence (5' to 3')	Purpose
AtALMT3 (At1g18420)	at1g18420-S2	GACACCCTGATGAGTTTTGC	RT-PCR, qRT-PCR
	at1g18420-A1	GGAAAGCCTGAAGAAGATGG	
AtALMT1 (At1g08430)	At1g08430-F	TTGAAGCGAGAGAGAAGGGGGACTA	RT-PCR, qRT-PCR
	At1g08430-R	CGAGCTCATTCTTCTTAATGGTGTT	
AtALMT6 (At2g17470)	At2g17470-F	CAGGTCCGCTATTCAATCTACAAAT	RT-PCR
	At2g17470-R	ACGCAGAACTTTTGCGCCTTCATTG	
AtALMT9 (At3g18440)	At3g18440-F	AAGAACTATGTCAAGCTCAGCGGC	RT-PCR
	At3g18440-R	CTTCAAAGAGAAGGTCGACTGGTCC	
AtALMT12 (At5g46600)	At5g46600-F2	ACTAAGCTCCAAGGCCTCTCTCGATG	RT-PCR
	At5g46600-R2	CGAGACTGTCGGAGAGAATCTCCGAC	
AtIPS1 (At3g09922)	AtIPS1-F	AGACTGCAGAAGGCTGATTCAGA	qRT-PCR
	AtIPS1-R	TTGCCCAATTTCTAGAGGGAGA	
UBQ10 (At4g05320)	UBQ10-F	GGCCTTGTATAATCCCTGATGAATAAG	RT-PCR
	UBQ10-R	AAAGAGATAACAGGAACGGAAACATAGT	
AtACT7 (At5g09810)	actin-F	TCTCTATGCCAGTGGTCGTA	RT-PCR, qRT-PCR
	actin-R	CCTCAGGACAACGGAATC	

4-4. Construction of binary plasmids and transformation of plants

For construction of plasmids, PCR was performed using high fidelity enzyme Prime STAR GXL DNA polymerases (Takara-bio). To amplify the 2,049 bp genomic sequence upstream of the longest ORF for *AtALMT3*, the primers were used as follows. Forward primer: 5'-ccgtcgacGAAACACTTGATAAAGCCACAAGTC-3' and reverse primers: 5'-gcgccggctctagaTTCTGGTTCTTGATTCCGATGATTGC-3' (capitals indicate the native sequence of AtALMT3; lower case letters indicate the adopter sequences). These primers include restriction-endonuclease-site sequences (underlined) and are designed for cloning to pENTR 3C entry plasmid (Invitrogen). The construction of the *AtALMT3* promoter::GUS reporter gene was performed using pGWB3 plasmid (Nakagawa et al. 2007) by the Gateway cloning system (Invitrogen). The *AtALMT3* open reading frame (coding sequence) of 1,743-bp (except for stop codon), were amplified using primers 5'-ccgtcgactctagaATGGCGGCACCAAAGCTGGAATC-3' and 5'-ccgcggccgcaaaggatcctccgccaccCTCAGAGACAGCTTCTTTG-3' which include restriction sites (underlined) and additional linkers (double underlined) for fusion of C-terminal GFP derived from the plasmid pTH2 (Chiu et al. 1996). Binary plasmid of the *AtALMT3* promoter fused to the coding sequence::GFP were modified from the *AtALMT3* promoter::GUS reporter gene plasmid using pGWB3 by replacing GUS gene with the AtALMT3::GFP fragments.

The binary plasmids were introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) or LBA4404. Transformations were performed floral dipping method as described previously (Clough and Bent 1998). Transgenic plants (T_1) were selected on Murashige-Skoog (MS) medium containing 20 μg/mL kanamycin and 100 μg/ml carbenicillin, and then grown in soil. The self-pollinated progeny (T_2) were selected by kanamycin resistant and analyzed for the gene expression, GUS staining and GFP fluorescence. GUS staining sample was observed on microscopes (SZ61 and CX31; Olympus, Tokyo, Japan).

Fluorescence was observed on All-in-One Fluorescence Microscope BZ9000 (Keyence, Osaka, Japan), with 488 nm excitation and 505 to 530-nm emission for GFP. For the staining of plasma membrane, plants were incubated with 20 mM FM4-64 (Molecular Probes) for 5 min, washed three times in water. Plates were scanned with excitation at 473 nm and detection with a 495- to 545-nm filter (green; GFP fluorescence) and a >560-nm filter (red; FM4-64) by confocal laser scanning microscope (FLUOVIEW1000-D BX-61; Olympus).

4-5. Soil culture and phosphorus analysis

A commercial Loamy soil was sieved through 2 mm holes. Three levels of phosphorus application treatments (0, 100, and 300 mg-P kg-soil^{-1} of Ca $(H_2PO_4)_2$•H_2O) were prepared. As nitrogen and potassium sources, 150 mg-N kg-soil^{-1} as $(NH_4)_2SO_4$ and 150 mg-K kg-soil^{-1} as K_2SO_4 were applied to all treatments. Seeds of Arabidopsis thaliana were directly sown on the 4.5 × 4.5 × 4.5 cm^3 plastic pots (10-20 seeds/pot) containing 50 g soil prepared below. After incubated 4˚C for 4 days for vernalization, plants were grown in a growth chamber under 16h/8h light period at 23˚C with 75 μmol cm^{-2} s^{-1}. After additional 25 d cultivation, the shoots of plants were cultivated. The shoots were oven-dried at 70˚C for 3 d. The dry matter of shoots was weighed, and then ground to a fine powder. Grind samples were digested with H_2SO_4-H_2O_2. The P concentration in the digested solution was quantified using the vanado-molybdate blue method (Murphy and Riley 1962).

4-6. Statistical analysis

All cultivations were replicated three times. Student's t-test and Tukey's test was performed using SPSS version 16J (SPSS Inc., Chicago, IL, USA). Significance was accepted at $P<0.05$.

The amino acid sequences were aligned by ClustalW program (Thompson et al. 1994) through a web server on DNA Data Bank of Japan (DDBJ;

http://www.ddbj.nig.ac.jp/). The phylogenetic tree was created by the NJplot software (Perrière and Gouy 1996).

Chapter 4

Characterization of citrate transporter induced by phosphorus deficiency

1. Introduction

Citrate secretion from plant roots into the rhizosphere plays an important role for mobilizing soil P. However, the citrate transporter induced by P deficient conditions is not identified yet. On the other hand, Al inducible multidrug and toxin extrusion (MATE) family citrate transporters; *HvAACT1* (Furukawa et al. 2007), *SbMATE* (Magalhaes et al. 2007) and *AtMATE* (Liu et al. 2009) have been reported. Citrate is the major molecule of the root-secreted carboxylates in P-deficient white lupin. P-deficiency induced MATE gene has been suggested as potential candidate for citrate secretion during P deficient white lupin, although the detail mechanism of organic acid secretion by P-deficiency is still unclear (Vance et al. 2003). Molecular approach is easier in model plant such as Arabidopsis rather than in specific plants. Therefore, in this chapter, we aimed to identify the transporter involved in citrate secretion on the P deficient conditions in *Arabidopsis thaliana*, which has also an ability to secrete citrate under P deficient conditions as shown in Chapter 2.

2. Results

2-1. Expression of *AtMATEs* under phosphorus deficient condition

First, the expression patterns of 56 *AtMATE* genes in both shoots and roots of *Arabidopsis thaliana* grown under +P and –P conditions were compared by using microarray data (Table 4-1). Seven and eight *AtMATEs*

Table 4-1 List of the gene expression of *AtMATEs* in the root and shoot of Arabidopsis detected by microarray analysis. Fold change (FC) means signal Intensity (SI) of –P/+P condition. Experiment 1 and experiment 2 indicate replications. Red and blue characters indicate more than double or less than half.

			Root						Shoot					
			Experiment1			Experiment2			Experiment1			Experiment2		
				SI			SI			SI			SI	
	Number	AGI code	FC	+P	–P	FC	+P	–P	FC	+P	–P	FC	+P	–P
AtMATE1	1	AT1G11670	1.16	5581	6487	1.67	4890	8156	1.30	550	713	1.06	568	537
AtMATE2	2	AT1G12950	3.32	795	2642	2.89	844	2440	1.13	197	222	0.74	171	233
AtMATE3	3	AT1G15150	1.10	326	358	1.32	339	447	0.91	427	388	0.91	395	436
AtMATE4	4	AT1G15160	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE5	5	AT1G15170	0.80	1140	916	0.94	1218	1147	1.01	1410	1430	1.03	1236	1199
AtMATE6	6	AT1G15180	0.94	335	315	1.09	326	355	0.63	5080	3209	1.63	6155	3784
AtMATE7	7	AT1G23300	1.73	782	1353	1.52	819	1249	1.04	162	169	1.29	222	172
AtMATE8	8	AT1G33080	0.56	999	559	0.50	1045	526	0.68	545	369	1.39	491	354
AtMATE9	9	AT1G33090	1.28	278	356	1.32	215	285	1.52	197	300	1.74	191	331
AtMATE10	10	AT1G33100	1.37	229	313	0.91	238	217	1.33	228	302	1.18	250	294
AtMATE11	11	AT1G33110	0.79	1107	871	0.71	1213	865	1.66	934	1551	1.80	955	1715
AtMATE12	12	AT1G47530	0.46	5056	2304	0.50	5996	2989	1.11	8429	9341	1.25	8586	10702
AtMATE13	13	AT1G51340	1.05	18859	19827	1.09	18393	20040	1.05	17090	17861	1.06	20285	21568
AtMATE14	14	AT1G58340	0.32	659	212	0.26	1003	257	0.72	279	200	1.39	163	226
AtMATE15	15	AT1G61890	0.73	11129	8087	1.35	7456	10038	1.82	7206	13147	1.21	7228	8746
AtMATE16	16	AT1G64820	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE17	17	AT1G66760	1.83	2225	4072	2.04	2050	4177	1.42	2162	3077	0.87	2194	1903
AtMATE18	18	AT1G66780	0.95	1892	1799	1.01	2244	2261	0.97	2115	2060	0.91	1822	1662
AtMATE19	19	AT1G71140	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE20	20	AT1G71870	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE21	21	AT1G73700	1.17	35	41	1.26	44	56	2.90	106	308	2.40	119	286
AtMATE22	22	AT2G04040	0.99	2288	2274	1.00	3143	3131	0.99	3859	3838	1.07	1776	1896
AtMATE23	23	AT2G04050	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE24	24	AT2G04066	0.82	117	96	1.01	133	133	0.88	117	103	0.83	119	99
AtMATE25	25	AT2G04070	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE26	26	AT2G04080	1.20	677	815	1.15	799	918	1.05	739	775	1.13	796	897
AtMATE27	27	AT2G04090	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE28	28	AT2G04100	1.36	935	1270	1.23	851	1051	1.27	218	276	1.72	192	330
AtMATE29	29	AT2G21340	1.17	1186	1389	1.06	1129	1195	1.28	3326	4244	1.15	3606	4146
AtMATE30	30	AT2G34360	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE31	31	AT2G38330	0.68	413	279	0.57	409	234	1.02	2588	2645	0.84	3170	2670
AtMATE32	32	AT2G38510	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE33	33	AT3G03620	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE34	34	AT3G08040	1.18	1251	1474	1.39	707	982	1.04	160	167	1.65	122	201
AtMATE35	35	AT3G21690	1.79	1470	2630	1.32	1598	2110	1.68	1592	2673	1.95	1383	2700
AtMATE36	36	AT3G23550	0.15	1640	253	0.26	1502	385	0.11	5361	596	0.63	2441	1541
AtMATE37	37	AT3G23560	0.81	1526	1230	0.76	1771	1345	1.16	980	1134	1.68	683	1146
AtMATE38	38	AT3G26590	1.40	486	681	1.34	440	592	2.04	146	297	2.52	86	217
AtMATE39	39	AT4G00350	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE40	40	AT4G21903	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE41	41	AT4G21910	1.58	9142	14478	1.75	8994	15755	1.42	5055	7160	2.00	4356	8713
AtMATE42	42	AT4G22790	1.78	630	1119	1.23	793	972	1.65	185	304	1.70	202	343
AtMATE43	43	AT4G23030	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE44	44	AT4G25640	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE45	45	AT4G29140	0.30	978	297	0.36	1076	388	0.75	818	616	1.06	732	777
AtMATE46	46	AT4G38380	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE47	47	AT4G39030	1.49	2095	3124	1.79	1498	2683	2.06	537	1106	2.33	375	876
AtMATE48	48	AT5G10420	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE49	49	AT5G17700	4.77	167	798	2.78	199	553	2.74	595	1632	2.71	508	1376
AtMATE50	50	AT5G19700	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE51	51	AT5G38030	1.77	410	723	1.71	340	581	1.05	179	187	1.21	55	67
AtMATE52	52	AT5G44050	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
AtMATE53	53	AT5G49130	1.00	20	23	1.00	23	19	1.00	28	30	1.82	29	52
AtMATE54	54	AT5G52050	0.86	9343	8061	1.15	7366	8462	2.80	9005	25245	1.25	7443	9266
AtMATE55	55	AT5G52450	1.38	2355	3258	1.15	2292	2645	1.64	1777	2917	2.12	1513	3213
AtMATE56	56	AT5G65380	2.29	2752	6294	1.37	3013	4120	0.57	6253	3542	0.63	6708	4233
negative control			1.10	20	25	1.11	28	20	1.10	21	33	1.16	29	19

were up-regulated by –P treatment in roots and shoots, respectively. *At1g12950* was accumulated only in roots and higher in –P than +P. *At1g12950* was highest induced among 7 *AtMATEs* genes up-regulated in –P roots. Transcripts for *At1g51340*, which has been reported as Al activated MATE (AtMATE), were found mainly in roots, but it was not regulated by P status. Therefore, At1g12950 was selected for the further analysis as a candidate of P deficiency responsive citrate transporter, and named AtMATE-PI1 (P-deficient Induced gene 1).

Deduced amino acid sequences of AtMATEs were compared to estimate the similarity of protein structures (Fig. 4-1). It was clarified that AtMATEs were highly varied and AtMATE-PI1 and AtMATE were classified different clades in the phylogenetic tree (Fig. 4-1). AtMATE-PI1 was closest to At1g61890, which was not regulated by P status.

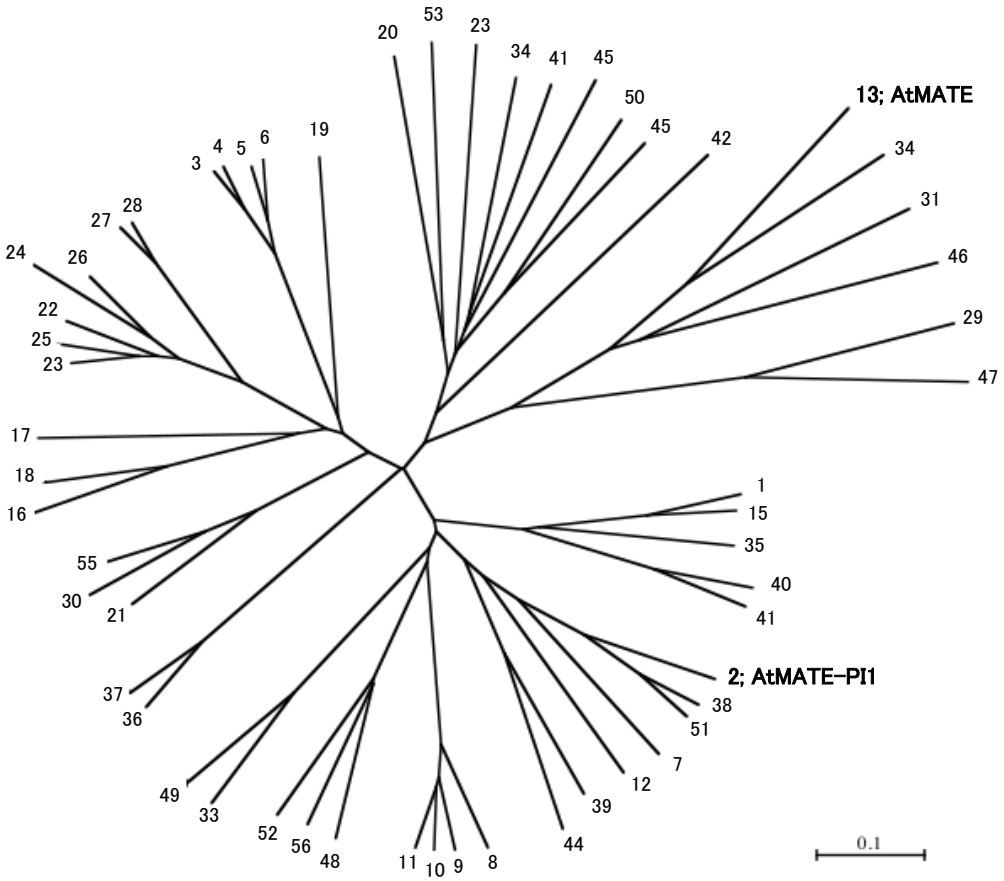

Figure 4-1 Phylogenetic relationship of AtMATEs proteins in *Arabidopsis thaliana*. The number is matched the number of Table 4-1. The amino acid sequences were aligned by ClustalW.

Since it is well known that the MATE family proteins transport not only citrates but also various other substrates (Omote et al. 2006), MATEs confirmed citrate transport activities or related properties in several plant species were used for similar analysis (Fig. 4-2). AtMATE-PI1 was closest to LaMATE from white lupin (*Lupinus albus* L.) among nominated MATEs from other species, although it had been reported that citrate transport activity was not detected for LaMATE by electrophysical analyses (Uhde-Stone et al. 2005).

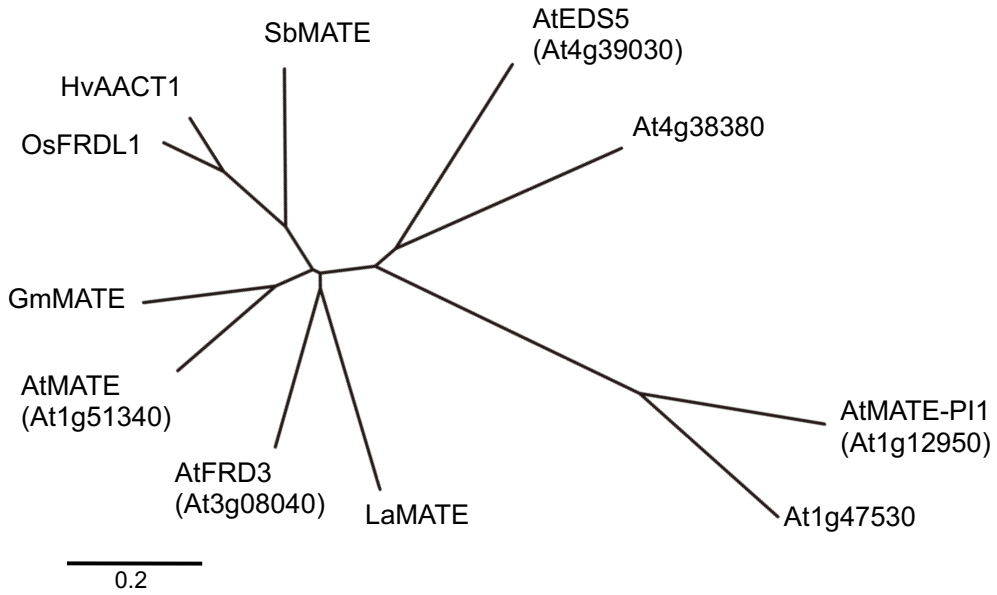

Figure 4-2 Phylogenetic relationship of AtMATE-PI1 proteins in Arabidopsis and different plant species containing GmMATE (Rogers et al. 2009), HvAACT1 (Furukawa et al. 2007), LaMATE (Uhde-Stone et al. 2005), OsFRDL1 (Yokosho et al. 2009) and SbMATE (Magalhaes et al. 2007). AtMATE-PI1 is low similarity (13%) to AtMATE, which was isolated Al activated citrate transporter. The amino acid sequences were aligned by ClustalW.

Quantitative RT-PCR revealed that mRNA of *AtMATE-PI1* was significantly up-regulated in -P than in +P condition, while that of *AtMATE* was not changed (Fig. 4-3).

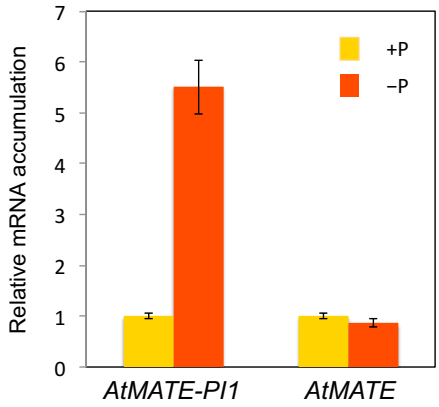

Figure 4-3 mRNA accumulation of *AtMATE-PI1* and *AtMATE*. qRT-PCR was performed for cDNAs of *Arabidopsis* roots with triplicate (error bar = SE). *AtMATE* are Al activated citrate transporter.

2-2. Loss-of-function analysis

To analyze the function of *AtMATE-PI1*, two *AtMATE-PI1* mutant lines (*atmate-pi1-1* and *atmate-pi1-2*, provided by Salk institute as CS859621 and Salk_136631, respectively) were obtained. T-DNA was inserted in intron and exon in *atmate-pi1-1* and *atmate-pi1-2*, respectively (Fig. 4-4).

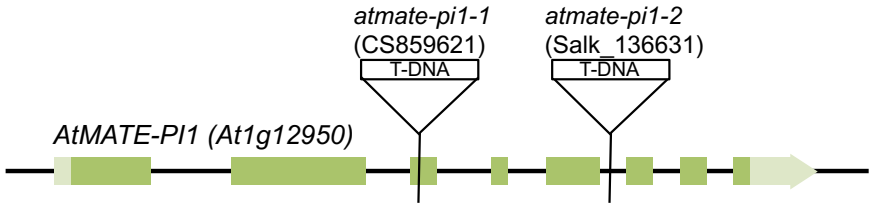

Figure 4-4 Gene structure of *AtMATE-PI1*. Two mutant lines were established and designated as *atmate-pi1* and *atmate-pi1-2*, which were inserted T-DNA in the exon and intron of *AtMATE-PI1*, respectively.

The mRNA for *AtMATE-PI1* was completely disappeared in both mutants (Fig. 4-5A). The exudation of both malate and citrate from -P plants was higher than +P plants in all lines (Fig. 4-5A). Citrate exudation decreased significantly by the knockout of *AtMATE-PI1* (Fig. 4-5B). The decreasing rate of citrate exudation in *atmate-pi1-1* and *atmate-pi1-2* was 30 and 35% in –P condition, and 63 and 47% in +P condition, respectively. There were no differences of malate exudation in three lines (Fig. 4-5B). These results suggested that AtMATE-PI1 was involved in the citrate exudation from Arabidopsis roots in normal condition and induced by P-deficiency.

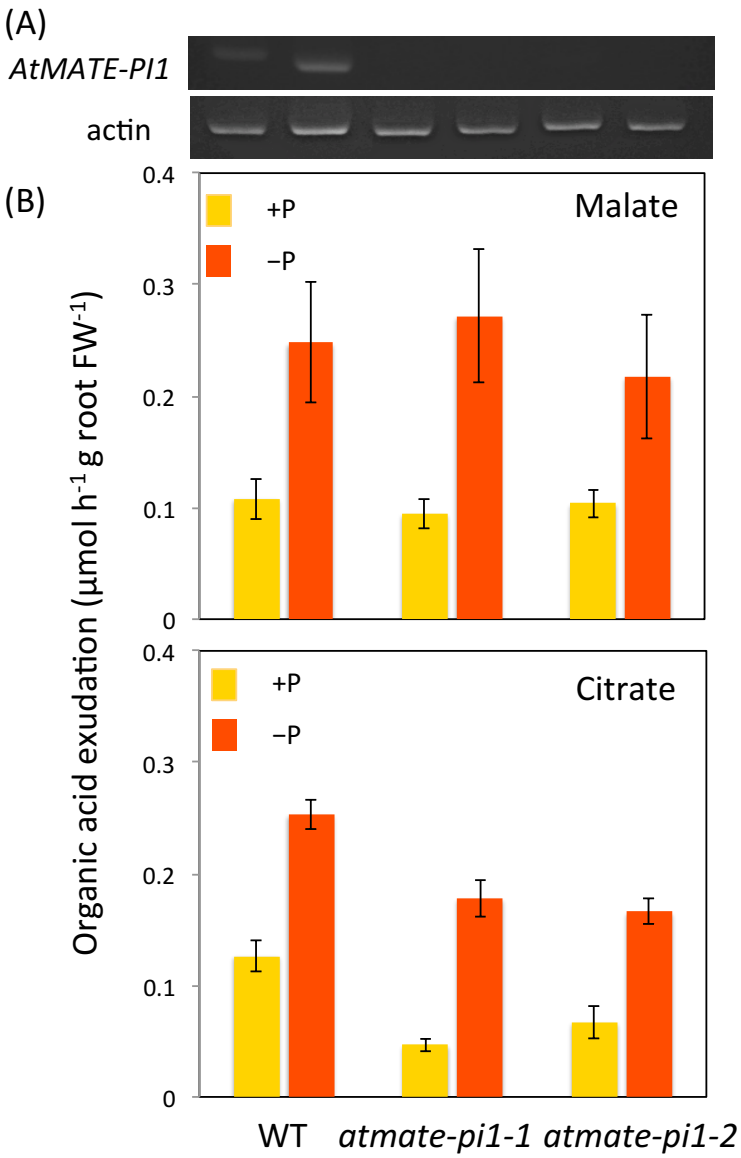

Figure 4-5 Effect of P nutrition on mRNA accumulation (A) and malate and citrate exudation from *Arabidopsis thaliana* roots (B). (*n* = 3, error bar = SE).

2-3. Histochemical localization of *AtMATE-PI1*

To investigate histochemical localization of *AtMATE-PI1* expression, I have generated transgenic *Arabidopsis thaliana* plants harboring a reporter gene, ß-glucuronidase (GUS), under the control of the putative *AtMATE-PI1* promoter (2,041 bp upstream from the start codon). *AtMATE-PI1* promoter::GUS lines showed expression in root hairs, especially in the adjacent region of the meristem of roots (Fig. 4-6).

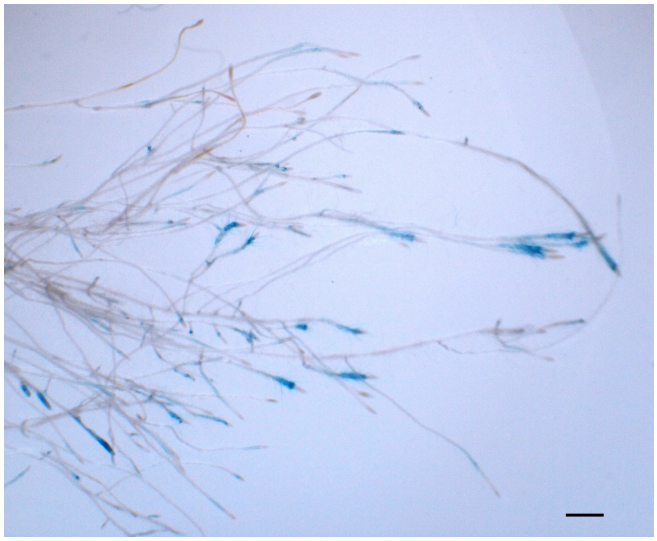

Figure 4-6 Localization of *AtMATE-PI1* promoter::GUS in transgenic seedlings. Transgenic plants carrying a *AtMATE-PI1* promoter::GUS construct were used for GUS staining after cultivated –P condition for 20 Days. (Bar=1mm)

3. Discussion

3-1. AtMATE-PI1 is involved in citrate transport under phosphorus deficiency in *Arabidopsis thaliana*

The loss of function analysis indicated that AtMATE-PI1 was involved in citrate exudation under P deficient condition in Arabidopsis (Fig. 4-5). Won et al. (2009) have reported that At1g12950 is one of the Root Hair-Specific Genes. WoLF PSORT program showed the score of subcellular localization for AtMATE-PI1 as below; plasma membrane 11, vacuole 1.0 and ER 1.0. This indicates that AtMATE-PI1 has a high possibility to locate in plasma membrane. *AtMATE-PI1* promoter::GUS lines showed expression in root hairs, especially in the adjacent region of the meristem of roots (Fig. 4-6). These facts support the hypothesis that AtMATE-PI1 is the citrate transporter located in plasma membrane of root hair cell and play a role for citrate transport from root to the rhizosphere under P deficient conditions.

Although the expression of AtMATE-PI1 was knocked-out in both *atmate-pi1-1* and *atmate-pi1-2* mutants, more than half of citrate exudation of them was remained in –P condition (Fig. 4-5). This indicated that there were other important transporter(s) involved in citrate exudation from root to the rhizosphere. In Arabidopsis, the MATE family comprises 56 genes (Table 4-1 and Fig. 4-1; Rogers and Guerinot 2002; Omote et al. 2006). They are involved in quite wide roles, such as lateral root formation (ALF5; Diener et al. 2001), iron homeostasis (FRD3; Rogers and Guerinot 2002), or disease resistance (EDS5; Nawrath et al. 2002). It was clarified that AtFRD3 played a role in iron uptake and was mainly expressed in the stellar tissues of Arabidopsis roots (Green and Rogers 2004). The microarray data indicated that AtFRD3 expression was not induced by P deficient condition (Table 4-1). However, transgenic plants overexpressing the AtFRD3 shows enhanced citrate efflux from the root (Durrett et al. 2007). This indicates that there is a possibility that AtFRD3 or other MATE transporters involved in citrate

efflux from roots. As the phylogenetic tree of AtMATE proteins, At5g38030 and At1g23300, which belonged to a clade containing AtMATE-PI1 (Fig. 4-1), At5g38030 and At1g23300 were predominantly expressed in roots and induced by –P (Table 4-1). Therefore, the two genes might be involved in citrate exudation under P deficient condition.

3-2. Perspectives

Shen et al. (2005) demonstrated that up-regulation of plasma membrane H^+-ATPase was associated with the secretion of citrate from soybean roots. Moreover, Tomasi et al. (2009) reported that burst of citrate exudation from cluster roots of P deficient white lupin depended on plasma membrane H^+-ATPase. In fact, it was suggested from transcriptomic analyses by using microarrays that some H^+-ATPase genes induced by P deficiency in root were presented (data not shown). Therefore, it is required to analyze the role of H^+-ATPases under P deficient condition.

The citrate transport activity could not be detected in this study. Electrophysiological analyses of AtMATE-PI1, the complementation analysis of the gene, and subcellular localization of AtMATE-PI1 will be provided to important information to understand detail functions of AtMATE-PI1.

4. Materials and Methods

4-1. Arabidopsis accessions

Arabidopsis thaliana (Col-0) was used for all of the control experiments. The T-DNA insertion mutants, *atmate-pi1-1* (CS859621), *atmate-pi1-2* (Salk_136631) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous plants were identified by PCR using appropriate primers (Table 4-2).

4-2. Plant material and growth condition

Surface-sterilized seeds of each Arabidopsis line were germinated in 10 x 10 cm plastic dishes containing sterile 1/2 media and 0.5% gellan gum (pH 5.6) for 4 days. Seedlings were transferred to same sized dishes containing 1/2 MS media with or without 0.625 mM phosphate. After 20 days, cultivated plants were transferred to inclined square dishes containing 2 mL distilled water in a corner of the dishes after washing with distilled water. The root exudates were collected for 6 h. Citrate and malate contents in the exudates were determined using the enzyme assay kits (E-kit; Roche Diagnostics). Fresh weight of root and shoot were weighted and immediately frozen in liquid N_2, and kept at -80˚C until use for RNA isolation.

4-3. Quantitative RT-PCR

Total RNA was extracted using the Agilent Plant RNA Isolation Mini kit (Agilent Technologies) as recommended by the manufacturer. Total RNA (300 ng) was reverse-transcribed into cDNA in a 10 μL reaction using the SuperScript® VILO™ cDNA Synthesis Kit (Invitorogen). The cDNAs and specific primer sets (Table 4-2) were used for qRT-PCR. Quantitative real-time RT-PCR was performed using an ABI StepOne real-time PCR system (Applied Biosystems) and SYBR Green kit (Takara-bio). The real-time PCR process comprised an initial denature at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, and a final dissociation stage of 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The data were analyzed using the ABI StepOne system software (Applied Biosystems). Transcripts were relatively quantified using actin transcripts as a control. Primer sequences for using qRT-PCR are shown in Table 4-2.

Table 4-2 List of primers used in this chapter.

Target gene	Primer name	Sequence (5' to 3')	Purpose
AtMATE-PI1 (At1g12950)	At1g12950-F2	TATTGTCTGGAGTGGCTGTG	RT-PCR, qRT-PCR
	At1g12950-R2	CCATTGAAGCCTCTTTCTCC	
	CS859621LP	TGGCGTGAGTTCTCTAACCAC	
	CS859621RP	AAAGCAAGATCATGGTCATGG	
AtMATE (At1g51340)	AtMATE-F	GCATAGGACTTCCGTTTGTGGCA	qRT-PCR
	AtMATE-R	CGAACACAAACGCTAAGGCA	
AtACT7 (At5g09810)	actin-F	TCTCTATGCCAGTGGTCGTA	RT-PCR, qRT-PCR
	actin-R	CCTCAGGACAACGGAATC	

4-4. Construction of binary plasmids and transformation of plants

For construction of plasmids, PCR was performed using Takara EX Taq (Takara-bio). To amplify the 2,041 bp genomic sequence upstream of the ORF for *AtMATE-PI1*, the primers were used as follows. Forward primer: 5'-GGCCAGATTTCTTCCTTGAC-3' and reverse primers: 5'-TCTCCGACGACAAGAGAAAG-3'. The PCR products sequences cloning to pCR8®/GW/TOPO® TA Cloning vector (Invitrogen) and the construction of the *AtMATE-PI1* promoter::GUS reporter gene was performed using pGWB3 plasmid (Nakagawa et al. 2007) by the Gateway cloning system (Invitrogen).

The binary plasmids were introduced into *Agrobacterium tumefaciens* strain LBA4404. Transformations were performed floral dipping method as described previously (Clough and Bent 1998). Transgenic plants (T_1) were selected on MS medium containing 20 μg/mL kanamycin and 100 μg/ml carbenicillin, and then grown in soil. The self-pollinated progeny (T_2) were selected by kanamycin resistant and analyzed for the gene expression, GUS staining. GUS staining sample was observed on a microscope (SZ61; Olympus).

4-5. Statistical analysis

All cultivations were replicated three times. Student's t-test and Tukey's test was performed using SPSS version 16J (SPSS Inc.). Significance was accepted at $P<0.05$.

The amino acid sequences were aligned by ClustalW program (Thompson et al. 1994) through a web server on DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/). The phylogenetic tree was created by the NJplot software (Perrière and Gouy 1996). Subcellular localization of the protein was predicted by the WoLF PSORT program (Horton et al. 2007; http://wolfpsort.org/).

Chapter 5

Organic phosphate mobilization by exogenous phosphatase and phytase

1. Introduction

Most plants respond to P deficiency by producing increased amounts of acid phosphatases (APases) in roots. Secretion of APase from roots is an important plant response to P deficiency. White lupin has an enhanced ability to secrete APase from roots under P-deficient conditions (Tadano and Sakai 1991; Wasaki et al. 2003). Ozawa and co-workers (1995) purified and characterized the APase protein, and cDNA has been isolated and named *LASAP2* (Wasaki et al. 2000). LASAP2 has wide substrate specificity, and is stable at pH 4.0–9.0 (Li et al. 1996, Miller et al. 2001).

When crude APase collected from white lupin exudates was injected into the rhizospheres of tomato and sugar beet plants, growth and P absorption of these plants increased (Tadano and Komatsu 1994). *LASAP2*-overexpressing tobacco has also shown a high ability to use organic P in sterile media (Wasaki et al. 2009). However, utilization of phytate in soil was not very high, although LASAP2 transformants had somewhat better organic P utilization compared to that of wild type (WT) tobacco.

Phytase, the phytate-specific phosphomonoesterase, is known to play an important role in phytate degradation. Because manipulation of the expression of phytase to improve phytate-P uptake from soil is an attractive strategy, many studies have inserted overexpression phytase genes of non-plants into plants to promote P uptake from soil (George et al. 2004, 2005b; Lung et al. 2006; Liu et al. 2011; Mudge et al. 2003; Richardson et al. 2001; Xiao et al. 2005, 2006; Yip et al.

2003; Zimmermann et al. 2003). However, clear effects of genetic modification have been shown only for soluble forms of P.

White lupin plants can use phytate-P in the soil (Adams and Pate 1992), although LASAP2 has a low specificity for phytate (Miller et al. 2001). Therefore, it has been hypothesized that root-secreted phytase of white lupin improves phytate-P mobilization in soils. Additionally, the adsorption capacity of organic phosphate reportedly shows the same tendency of the adsorption capacity of inorganic phosphate (Turner et al. 2002). Therefore, it can be speculated that effects of phosphatase and phytase on P mobilization are high in low P-adsorptive soils. Recently, a phytate specific phosphatase was isolated from white lupin (Kaneko 2004), although characteristics were still unknown.

This chapter aimed to investigate the effect of *LASAP3* overexpression on utilization of organic P in soils by tobacco plants. First, it was investigated the roles of APase homologs of white lupin based on their expression analysis. The *LASAP3* overexpression line was established and used for soil culture with a *LASAP2*-overexpressing line and wild type. Additionally, the effects of white lupin-derived phosphatase and phytase overexpression on P accumulation from two soils with differing P adsorption capacities were investigated.

2. Results

2-1. Gene expression of *LASAP1, 2 and 3* for *Lupinus albus* under P deficient condition

P acquisition strategy of low P tolerant plant, white lupin, is well investigated, such as the function of root-secreted APase, designated as LASAP2, and other homologs, LASAP1 and LASAP3 (Wasaki et al. 1999, 2003). LASAP2 is a major player to enable P from organic P in the rhizosphere (Wasaki et al. 2000, 2009), however, roles of others were still not well explained. Therefore, the

expressions of the APase homologs were characterized here.

The data of qRT-PCR for *LASAP1*, *LASAP2* and *LASAP3* were shown in Table 5-1. *LASAP1* was constitutive but up-regulated to some extent by –P in all tested organs, especially in roots. The amount of *LASAP1* mRNA in senescent leaves was higher than in younger leaves. Accumulation of LASAP1 mRNA in cotyledons, flowers and pods was found but not very high. Expression of *LASAP2* gene was specific in roots under –P conditions. mRNA for *LASAP3* was most abundant in ripening seeds. Accumulation of *LASAP3* mRNA was much in immature leaves, whereas it was less in older leaves, stems and roots (Table 5-1).

Table5-1 mRNA accumulation for *LASAP1, LASAP2 and LASAP3*, analyzed by qRT-PCR. (a) Germinating stage, (b) Vegetative growth stage and (c) Flowering and grain-filling stage.
Over 500 pg / µg-total RNA are indicated by red characters.

Site	Gene expression (pg / µg-total RNA)					
	LASAP1		LASAP2		LASAP3	
(a) Germinating stage						
Germinating seeds	5.25		0.03		8.46	
Unexpanded leaves	127.75		0.18		82.25	
Stems	249.38		0.09		94.50	
Seedling roots	514.51		2.15		164.50	
Cotyledons	96.25		0.23		29.75	
(b) Vegetative growth stage						
	+P	-P	+P	-P	+P	-P
Leaves	86.19	260.54	0.30	12.40	22.75	37.00
Stems	158.60	490.39	0.20	47.79	62.13	120.15
Roots	189.88	449.10	0.28	790.59	47.25	78.30
Other roots		1234.93		542.96		123.23
Cluster roots		1270.52		1484.84		108.12
(c) Flowering stage						
Fully expanded leaves	67.38		0.07		14.00	
Unexpanded leaves	107.33		0.27		205.24	
Yellow leaves	189.46		0.30		62.98	
Flowers	88.98		0.26		223.43	
Young pods	220.88		0.21		505.90	

2-2 Identification of a *LASAP3*-overexpressing tobacco line

The cDNA of *LASAP3* was amplified by PCR and introduced into tobacco plants under control of the CaMV35S promoter. After transformation, two kanamycin-resistant lines (lines 1 and 3) of tobacco plants were selected. Quantitative RT-PCR for T1 plants revealed that line 3 plant showed about three times higher expression of LASAP3 in roots than line 1 showed (data not shown). The T2 seedlings were tested for a Mendelian segregation ratio of 3:1. The homozygous line 3-3 (35S-LASAP3) was established and used for experiment of APase activity. The GmPhy7 sequence exhibited a high similarity to a class of metallophosphoesterases, which show wide substrate specificities (Hegeman and Grabau 2001). Therefore, the APase activity was analyzed instead of phytase. APase activity of root exudate from 35S-LASAP3 plant was significantly higher than that from WT plant at pH 5.5 condition and was almost identical to that from 35S-LASAP2 (Fig. 5-1).

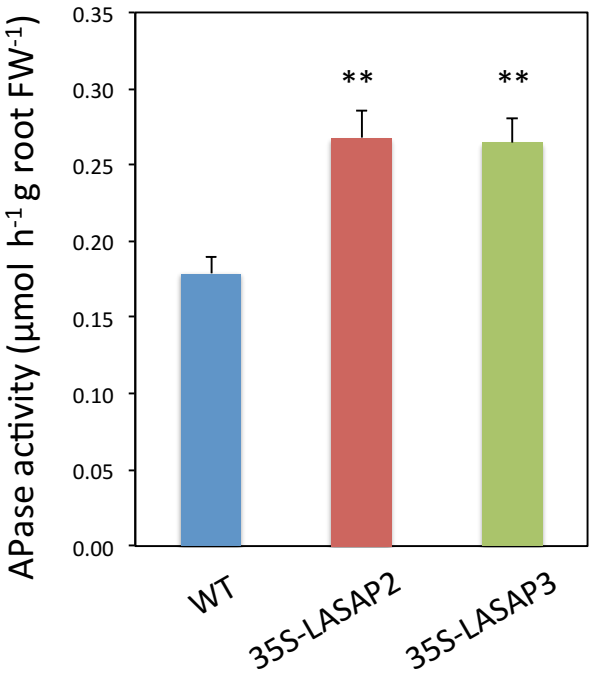

Figure 5-1 APase activity of root exudates of tobacco plants. Data are presented as the mean ± standard error (*n*=5). Values followed by asterisks are significantly different from the WT according to Dunnett test results (***P*<0.01).

2-3. Soil analysis

Two different type soils were used for plants cultivation. Properties of the two soils are presented in Table 2. Soil pH (1:2.5 of H_2O) of the Andosols and Regosols was, respectively, 5.2 and 5.6 (Table 5-1). Regosols show a

comparatively low P absorption coefficient (60.9 ± 11.7 mg-P_2O_5/ 100g soil) compared to that of Andosols (808.5 ± 6.8 mg-P_2O_5/ 100g soil). Total P in Andosols (247.4 ± 19.9 mg-P/ kg soil) was about three times higher than that in Regosols (89.6 ± 2.9 mg-P/ kg soil). However, available P in Andosols (13.4 ± 0.7 mg-P/ kg soil) was about one-half of that in Regosols (20.5 ± 2.3 mg-P/ kg soil).

Table 5-2 Properties of used soils.

Soil type	Texture	pH (H_2O)	P absorption coefficient (mg-P_2O_5/ 100g soil)	Total-P (mg-P/ kg soil)	Truog-P (mg-P/ kg soil)
Andosols	Loam	5.2	808.5 ± 6.8	247.4 ± 19.9	13.4 ± 0.7
Regosols	Sandy loam	5.6	60.9 ± 11.7	89.6 ± 2.9	20.5 ± 2.3

2-4. Growth and P accumulation of plants in Andosols

Fig. 5-2A shows the plant growth on the Andosols at 49 d after transplanting. The growth of both overexpression lines was increased significantly compared to WT in +Po and +Pi treatment. The 35S-LASAP2 and 35S-LASAP3 lines showed similar trends, although the accumulated dry weight of the 35S-LASAP3 line was slightly higher than that of the 35S-LASAP2 line.

The P contents of plants grown on Andosols are portrayed in Fig. 5-2B. The P uptake by both overexpression lines was higher than that of the WT in all treatments, similar to the trend shown for plant growth. The P contents of the 35S-LASAP2 lines were increased by 33%, 17%, and 52%, respectively, in No P, +Po, and +Pi treatments. The P contents of 35S-LASAP3 lines were increased by 30%, 19%, and 75%, respectively, in No P, +Po, and +Pi treatments. The P contents of 35S-LASAP3 lines were slightly higher than those of the 35S-LASAP2 lines in the +Po treatments. However, no significant difference was found between the two lines.

2-5. Growth and P accumulation of plant in Regosols

Fig. 5-3A shows the plant growth on the Regosols at 42 d after transplanting. The growth of each overexpression line was greater than that of WT in No P treatment, although no significant differences were found from either the +Po or +Pi treatment.

The P contents of the plants are presented in Fig. 5-3B. The P uptake by 35S-LASAP2 and 35S-LASAP3 lines was higher than that of the WT in all treatments. The P contents of 35S-LASAP2 lines were increased by 114%, 34%, and 2%, respectively, in No P, +Po, and +Pi treatments. The P contents of 35S-LASAP3 lines were increased by 59%, 21%, and 4%, respectively, in No P, +Po, and +Pi treatments.

2-6. P uptake efficiency of three tobacco lines under low available P conditions

Fig. 5-4 shows the relation between plant growth and P contents in the shoots grown in the low available P (No P and +Po) treatments. In all plants, positive correlation was found in plants grown in Regosols (r^2 = 0.96–0.99), although weaker correlation was found in plants grown in Andosols (r^2 = 0.48–0.88). The slope, which indicates the P uptake ability of plants from soils, was higher in Regosols than in Andosols for all three lines. The slopes of 35S-LASAP2 (0.0058) and 35S-LASAP3 (0.0050) were higher than WT (0.0044) in Regosols. This result indicates that *LASAP2* and *LASAP3* overexpression can improve the acquisition of organic P from soils, especially in Regosols.

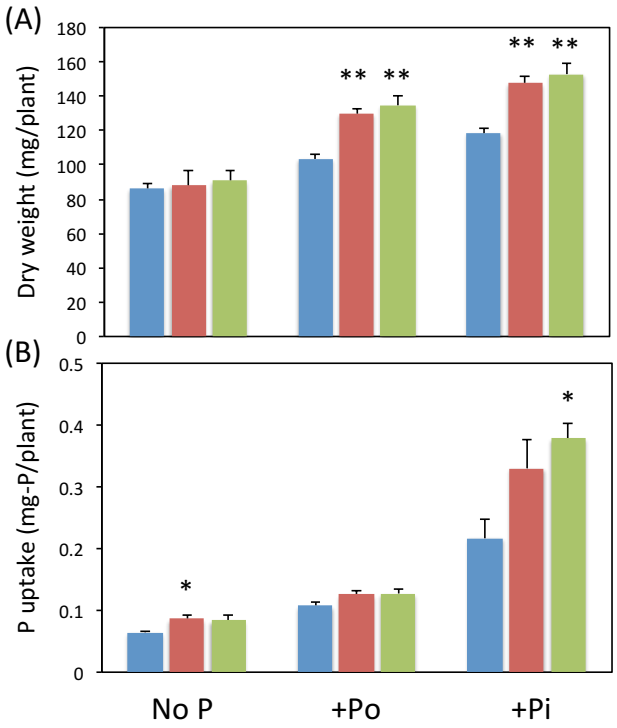

Figure 5-2 Growth (A) and P uptake (B) of the tobacco plants cultured in Andosols. Blue, red, and green bars respectively show wild type (WT), 35S-LASAP2, and 35S-LASAP3 lines. Data are presented as the mean ± standard error (*n*=5). Values followed by asterisks are significantly different from those of WT according to Dunnett test results (**P*<0.05; ***P*<0.01).

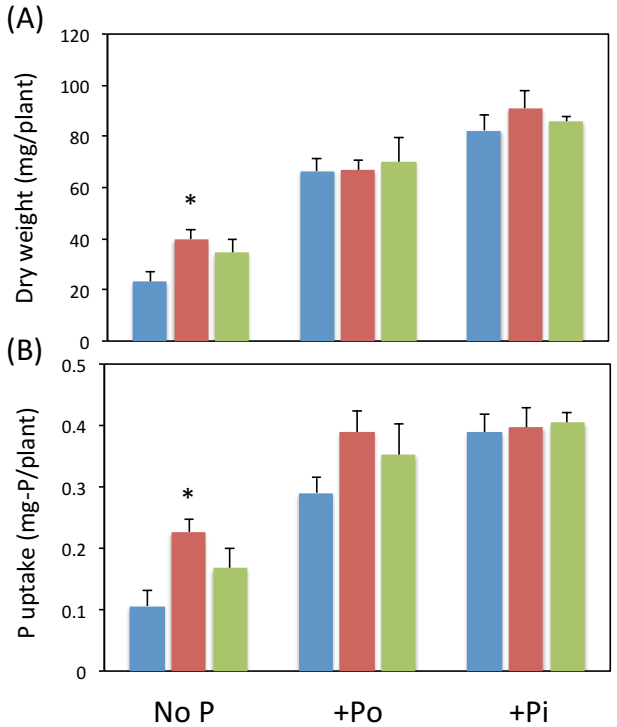

Figure 5-3 Growth (A) and P uptake (B) of tobacco plants cultured in Regosols. Blue, red, and green bars respectively show wild type (WT), 35S-LASAP2, and 35S-LASAP3 lines. Data are presented as the mean ± standard error (*n*=5). Values followed by asterisks are significantly different from WT according to Dunnett test results (**P*<0.05).

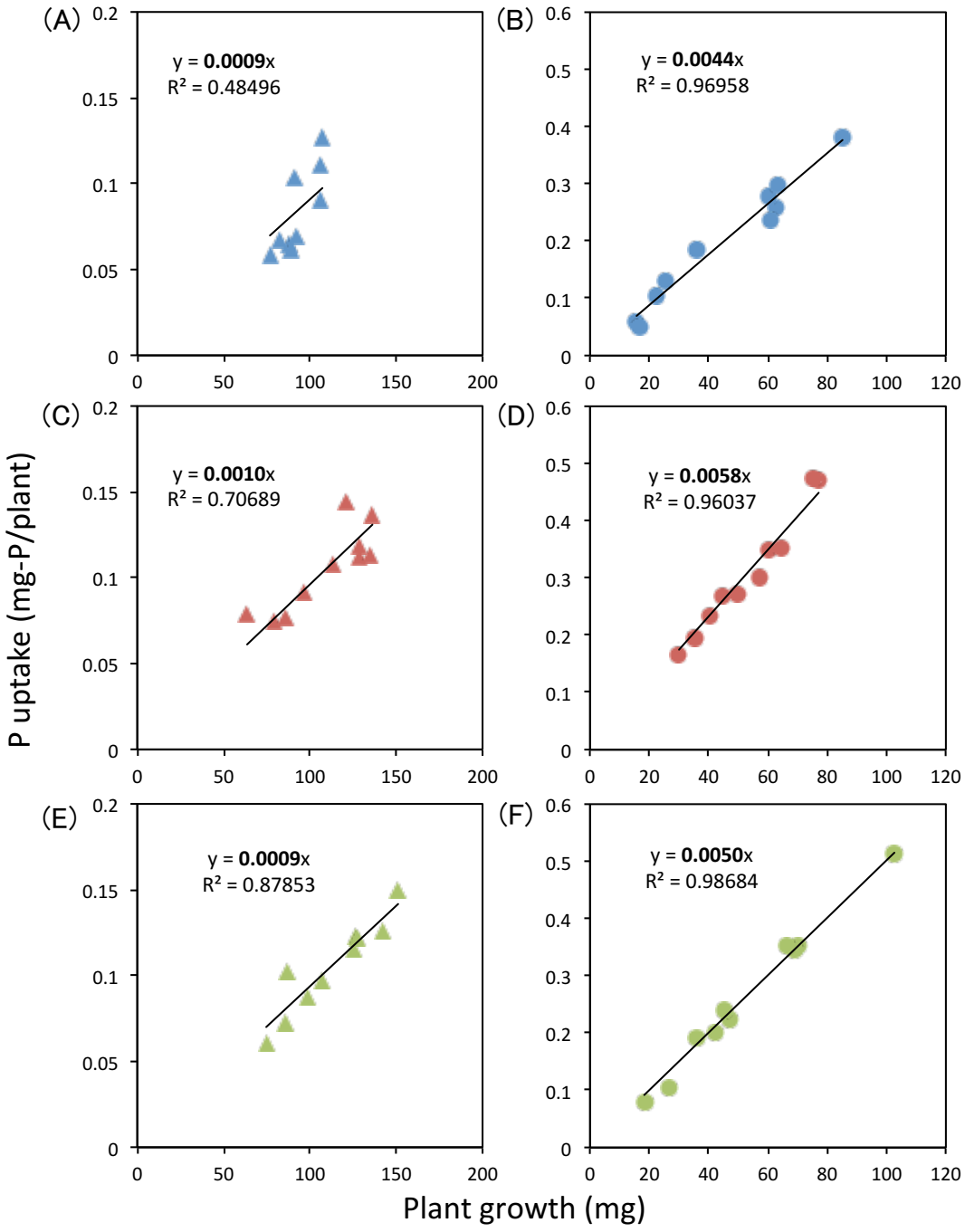

Figure 5-4 Relation between dry weight of plants and P content of shoots of WT (A,B), 35S-LASAP2 (C,D) and 35S-LASAP3 (E,F). Circle and triangle symbols respectively denote data of Regosols and Andosols.

3. Discussion

3-1. Role of three APase homologs of white lupin under P deficient condition

Quantitative mRNA accumulation of *LASAP1, 2 and 3* was analyzed to understand the roles of APase homologs of L. albus under P deficient condition. At first, mRNA accumulation of *LASAP2* was most abundant in the cluster roots formed under -P conditions (Table 5-1). These data were consistent with results in previous studies (Wasaki et al. 2003, 2008). Deduced amino acid sequences for LASAP1 and LASAP2 were similar to AtPAP12, 10 and 26 (Fig. 2-4). Tran et al. (2010) reported that AtPAP12 and AtPAP26 are the predominant purple acid phosphatase (PAP) isozymes secreted by P deficient Arabidopsis root. Moreover, Hurley et al. (2010) reported that AtPAP26 is the principal contributor to Pi stress inducible APase activity, and that it plays an important role in the Pi metabolism of P deficient Arabidopsis. In addition, Wang et al. (2011) investigated and concluded that AtPAP10 was induced by Pi limitation at both transcriptional and posttranscriptional levels and associated with the root surface after secretion. Moreover, they suggested that AtPAP10 played an important role in plant tolerance to Pi limitation. These facts support the hypothesis that LASAP1 and LASAP2 are strongly involved in Pi metabolism and P uptake by secretion from roots. The deduced amino acid sequence of LASAP3 was closest to AtPAP15 among 29 PAPs of Arabidopsis (Fig. 2-4). It has been reported that AtPAP15 has phytase activity and hydrolyzes phytate to myo-inositol and free Pi (Zhang et al. 2008; Kuang et al. 2009). AtPAP15 might be not secreted from root, although the mRNA accumulation was in the root. However, it was suggested that primary structure of LASAP3 was secretory type protein based on the Target-P program (data not shown). Thus, LASAP3 may play a role for release P from phytate in the rhizosphere.

3-2. Organic phosphate mobilization by exogenous phosphatase and phytase

A transgenic tobacco line was established overexpressing *LASAP3* gene, which is the lupin ortholog of phytase isolated from germinating soybean (GmPhy7). It has been suggested that GmPhy7 shows hydrolyzing activities not only to phytate but also other general organic phosphate compounds (Hegeman and Grabau 2001). In our experiments, the APase activity in root exudates of 35S-LASAP3 was significantly higher than WT (Fig. 5-1). The result suggests that 35S-LASAP3 line has higher ability to secrete APase than the WT of tobacco. Moreover, it was concluded that LASAP3 could have activities not only of APase but also phytase. Several genes for APase having phytase activity have been identified from some plants such as soybean, *Medicago truncatula*, and tobacco (Hegeman and Grabau 2001; Xiao et al. 2005; Lung et al. 2005).

In the present experiment, the effect of *LASAP2* and *LASAP3* overexpression on the growth of tobacco plants in soil of two types under different P conditions was evaluated and compared to that of wild type of tobacco. Results clarified that growth and P accumulation of both 35S-LASAP2 and 35S-LASAP3 lines showed similar trends and were higher than those of WT (Figs. 5-2 and 5-3). In our previous study, the potential for the *LASAP2*-overexpressing tobacco to increase organic P in a brown lowland soil from a -P plot from the long-term experimental field of Hokkaido University was evaluated (Wasaki et al. 2009). Growth and P acquisition of *LASAP2* transgenic plants were higher than those of WT. These results suggest that both *LASAP2* and *LASAP3* overexpression can improve the acquisition of organic P from soils.

Regosols show quite low P absorption coefficients compared to those of Andosols (Table 5-1). The P uptake of both overexpression lines from +Po in Regosols was higher than that of WT. However, the increase of P uptake from +Po in Andosols was low (Figs. 5-2 and 5-3). Moreover, the P uptake efficiency of all three lines grown under low available P conditions was higher in Regosols than in Andosols (Fig. 5-4). These results support the hypothesis that the solubility of

organic P is a limiting factor for mobilization in the soil, as inferred from results of other studies (George et al. 2005a; Wasaki et al. 2009).

It was expected that 35S-LASAP3 plants would show a much greater effect on increases of growth and P accumulation than 35S-LASAP2 plants in +Po treatment. The results of 35S-LASAP2 and 35S-LASAP3 plants showed a similar trend, but 35S-LASAP2 plants showed increased P accumulation in +Po of Regosols. For Regosols, the P content of WT in the +Po treatment was higher than that in the No P treatment (Figs. 5-2 and 5-3). This result might derive from the fact that tobacco native secreted APase (NtPAP), which also had phytase activity (Lung et al. 2008). Therefore, the effect of APase and phytase overexpression on the improvement of phosphorus use ability can be thought to depend on the difference of the substrate specificities to existing organic P that is originally present in the soil.

Phytate mostly exists in soils in an unavailable form such as Fe-Phytate or Al-Phytate (Turner et al. 2002). For effective use of these forms, chelation by organic acids is important (George et al. 2005a; Wasaki et al. 2009). Adams and Pate (1992) reported that white lupin plants can use phytate-P in the soil. White lupin is well known to form a unique root structure under P-deficient conditions to mobilize unavailable P forms, so-called cluster roots (Neumann and Martinoia 2002). Reportedly, huge amounts of organic acids and phosphatases were secreted from the cluster roots of white lupin under P deficient conditions (Tadano and Sakai 1991; Neumann et al. 1999; Wasaki et al. 2003). Further improvement in phytate-P mobilization is expected from the tandem genetic manipulation of phytase and a key protein for exudation of organic acids, such as citrate or malate. Elucidation of key functions necessary for organic acid exudation from roots under low P conditions is also anticipated in the near future.

It is equally important to investigate the interactions of microorganisms in the rhizosphere. Doolette et al. (2010) reported that phytate was decomposed rapidly by soil microorganisms to other organic phosphates, meaning that phytate

is not highly stable, but that it might be a biologically available form of P. Exudation of organic acids by plants is also closely involved in the dynamics of sparingly soluble P and interactions with soil microorganisms. Dynamics and effects of microorganisms on P in the soils should be considered carefully.

4. Materials and Methods

4-1. Quantitative RT-PCR for *LASAP1*, *LASAP2* and *LASAP3*

White lupin was cultured in a nutrient solution containing 0 or 64 μM P according to Ozawa et al. (1995). Plants were separated into roots, stems and leaves at 7 days after germination and 23 d after P treatment (34 day after geramination) and immediately frozen in liquid N_2. The roots of plant grown 0P condition were separated three parts. Total RNA was extracted from samples using an SDS–phenol method (Palmiter 1974). First-strand cDNA was prepared using a Super Script Ⅲ First-Strand Synthesis System for RT-PCR (Invitrogen) and used as templates for quantitative RT-PCR. Fragments for *LASAP1*, *LASAP2* and *LASAP3* cDNA was amplified by Smart Cycler Ⅱ System Takara-bio SC200N using specific primers (Table 5-3) as follows conditions: 95°C for 5 s and 60℃ for 20 s for 45 cycles (for *LASAP1* and *LASAP2*), 95°C for 5 s, 57℃ for 20 s and 72℃ for 15 s for 50 cycles (for *LASAP3*).

Table5-3 List of primers used in this chapter.

Target gene	Sequence (5′ to 3')	Primer name	Tm	length(bp)
LASAP1	TGTATTGGTGTTTTGCGATGGAGG	cSAP1rt-S2	58.8	175
	GGAAAGCAGTGATTGTGTCATGG	cSAP1rt-A2	58.7	
LASAP2	AGTAGTTTTGTTGCAATAGCTT	SAP2-RT5S	47	116
	GCCAGTGGATATGCCACTTGATAG	SAP2G-A2	57	
LASAP3	CCCTCATTACAAGCCATGAG	LaPhy-S5	56.3	154
	CACCTGACTAGTAATAAGCCTG	cSAP3rt-A	56.7	

4-2. Transformation of *Nicotiana tabacum*

The PCR product of the full-length LASAP3 cDNA was cloned into the pCR8®/GW/TOPO® TA Cloning vector (Invitrogen) and sequenced. The fragment was cloned downstream of the CaMV35S promoter of the pK7WG2D vector using the Gateway system according to the manufacturer's instructions (Invitrogen). The construct was transferred to the *Agrobacterium tumefaciens* LBA4404 strain using an electroporation system (Gene Pulser Xcell; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Tobacco (*Nicotiana tabacum* L. cv. SR1) plants were transformed with LASAP3 cDNA using Agrobacterium-mediated transformation (Horsch et al. 1985). Transformation of LASAP3 was checked by PCR amplification of the full-length *LASAP3* cDNA using genomic DNA of the T_1 generation. A 3:1 segregation test of T_2 seeds for kanamycin resistance was conducted for 2 weeks. The lines of all surviving plants were used for additional experiments. A transgenic tobacco line expressing *LASAP2* under control of the CaMV35S promoter (Wasaki et al. 2001) was also used.

Roots of T_1 tobacco plants were used for total RNA extraction. Plants were grown under hydroponic culture conditions for about 1 month in a growth chamber with a 16/8 h day/night cycle, light intensity of 300 μmol m^{-2} s^{-1}, and a 25/20°C day/night temperature regime with relative humidity of 60%. The hydroponic culture solution components were identical to those used in a previous study (Ozawa et al. 1995). Total RNAs of tobacco plants were isolated using an SDS–phenol method. The RNA was treated with DNase I (RT grade; Roche Diagnostics) at 37°C for 30 min to digest contaminating genomic DNA; then it was reverse-transcribed using the Superscript III first Strand cDNA Synthesis Kit for RT-PCR (Invitrogen). The first strand cDNAs and specific primers for LASAP3 (LASAP3-S7: 5'-AGCATAGACATTCTGTTCCT-3' and reverse primers: 5'-GATAGTGTGTGTGACATTCA-3') were used as templates for quantitative reverse transcription PCR using a SmartCycler™ II System (Cepheid, Sunnyvale, CA, USA), which was run as follows: 94°C for 5 s, 57°C for 20 s and 72°C for 15 s

for 50 cycles.

4-3. Measurement of acid phosphatase activity of root exudate

Tobacco seeds were bathed in a sodium hypochlorite solution (0.5% available chlorine) with agitation for 1–2 min. Surface-sterilized seeds were washed thoroughly three times in sterilized water and germinated in 10 × 10 cm plastic dishes containing sterile media with 1/2 MS media and 0.5% gellan gum (pH 5.6) for 14 days. Three plants were transferred to another 1/2 MS media dish and cultivated vertically. At 21 days after transplanting, plant roots were transferred to inclined square dishes containing 2 mL distilled water in a corner of the dish after washing of the roots with distilled water. The root exudates were collected for three hours, and the plant root fresh weight was weighed.

APase activity of root exudates was measured according to the method described by Wasaki et al. (2005) with minor modification using a fluorogenic substrate, 4-methylumbelliferyl phosphate, and a microplate reader (ALVO X1; PerkinElmer, Inc., MA, USA). Substrates were dissolved with dimethylsulfoxide (DMSO) as a solvent and diluted to 1 mM with the 0.1 M 2-(N-morpholino)-ethanesulfonic acid (MES) buffer (pH 5.5). Then 20 μL of the root exudate solution was mixed with 80 μL of the MES buffer and 100 μL of the substrate solution in 96-well microplates. Fluorescence was measured for 1 h at 30°C with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Fluorescence readings were performed every 2 min. Results were expressed as the increasing rate of 4-methylumbelliferone liberation per root FW (μmol h^{-1} g root FW^{-1}).

4-4. Soils for pot culture experiments

Andosols

A commercial soil containing no fertilizers and deficient in microorganisms (Hokkai Sankyo Co., Ltd., Hokkaido, Japan) was sieved through 2 mm screens. Three plots were prepared as follows: No P (0 mg-P kg-soil^{-1} of Ca $(H_2PO_4)_2 \cdot H_2O$), +Pi (100 mg-P kg-soil^{-1} of Ca $(H_2PO_4)_2 \cdot H_2O$) and +Po (100 mg-P kg-soil^{-1} of inositol hexakisphosphate, dodecasodium salt; Sigma, St. Louis, MO, USA). As nitrogen and potassium sources, 150 mg-N kg-soil^{-1} as $(NH_4)_2SO_4$ and 150 mg-K kg-soil^{-1} as K_2SO_4 were applied to all plots. Pots were filled with approximately 100 g of soil and deionized water was added to 60% of water holding capacity.

Regosols

A field soil at Hiroshima University was sieved through 2 mm holes and mixed with 10% (w/w) peat moss. Three plots were prepared similarly to preparations used for Andosols. Pots were filled with approximately 250 g of soil and deionized water was added to 50% of water holding capacity.

Soil analysis

Part of prepared soils in above was used for soil analysis. Soil pH was measured in a water suspension (ratio 1:2.5). The P absorption coefficient was analyzed according to methods described in an earlier report (Sekiya 1970). The total P concentration of soil was determined using the vanadomolybdate blue method (Murphy and Riley 1962) after digestion of the sample using H_2SO_4-H_2O_2. The available P concentration was analyzed as Truog-P (Truog 1930).

4-5. Growth conditions of pot culture

Tobacco seeds of the wild-type (WT), a *LASAP2* transgenic line (35S-LASAP2), and a *LASAP3* transgenic line (35S-LASAP3) were sterilized as preparatory procedures. The seeds were sown in 1/2 MS medium containing 3.0% (w/v) sucrose and 0.8% (w/v) agar. After germination for 14 days, the seedlings were transferred to pots. Three plants were transferred per pot and cultured in a growth chamber with a 16/8 h day/night cycle, light intensity of 300 μmol m^{-2} s^{-1}, a 25/20°C day/night temperature regime with relative humidity of 60%. To maintain the soil water concentration, deionized water was supplied every 2–3 d. Plant shoots were collected at 49 d (on Andosols) and 42 d (on Regosols) after sowing. They were weighted after oven-dried at 70°C for 3 d. An aliquot of approximately 80 mg for each sample was weighed and digested using H_2SO_4-H_2O_2. The P concentration in the digested solution was quantified using the vanadomolybdate blue method (Murphy and Riley 1962).

4-6. Statistical analysis

For this study, five pots (five samples) were prepared for each soil treatment. Plants from each pot were analyzed individually. Significant differences were inferred using general analysis of variance (ANOVA). Gene modification means were compared using Dunnett tests (SPSS ver. 16; SPSS Inc.).

Chapter 6

General discussion

For developing sustainable agriculture system, reducing consumption of finite P resource, we should increase the efficiency of P uptake from arable lands to crop plants via ability of mobilizing unavailable P in soils. Therefore it is important to elucidate the P mobilizing mechanism in soil by plants. In this study, *Arabidopsis thaliana* and *Lupinus albus* were used for understanding the detail mechanisms of P mobilization. In this study, I obtained two main results; 1) novel transporters for malate and citrate exudation induced by P deficient *Arabidopsis thaliana* were characterized, 2) solubility of soil P is important for the mobilization of organic P by root-secreted enzymes. Here, I will discuss about these results in this study as below.

1. Impacts of organic acids in root exudates induced by P deficient condition on P dynamics and microbial communities in the rhizosphere

In P deficient conditions, it has been known that plants secrete various metabolites, such as organic acids, amino acids and sugars (e.g. Raghothama and Karthikeyan 2005). Arabidopsis also increased the exudation rate of organic acids, especially glyceric acid, pipecolic acid, citric acid, threonic acid and malic acid (Table 2-1). Malate and citrate are the common organic acids of root exudates induced by P deficiency. Jones (1998) reported in review paper that the most of organic acids in the rhizosphere will be rapidly adsorbed to the soil's exchange phase while the resultant concentration in the soil solution will be in the range of 1-100 μM depending on Al and P stress level. The concentration of malate and

citrate from the Arabidopsis were quite low in this study, however, the P uptake ability from soil was lower in mutants of malate secretion than WT. Therefore, it is concluded that organic acid secretion from root is important for P mobilization in soils.

The rhizosphere was defined as a specific region affected by high microbial densities (Hiltner 1904). Organic acids in root exudates may have strong impacts on the microorganisms, including phosphate-solubilizing bacteria, in the rhizosphere. However, little is known about the effect such as changing the microbial community structure. Marschner et al. (2002) analyzed the effect of organic acids for the community structure in the rhizosphere of cluster roots of lupin by PCR-DGGE. They concluded that the microbial community structures were dependent on the kind of organic acids. Wasaki et al. (2005) also obtained similar results in the experiment to investigate the effects of elevated CO_2 on the microbial community structure in the rhizosphere of white lupin. It was suggested by PCR-DGGE and functional analyses that the age of cluster roots of white lupin had the strongest effect on the microbial community structure.

The effects of microbes in the rhizosphere on P mobilization must not be negligible, although it was not estimated in this study. Soil microorganisms are mediating P availability with multiple ways, as reviewed by Richardson and Simpson (2011). The effects of root exudates, such as organic acids as carbon sources and minor organic acids and secondary metabolites as the signal molecules on the microorganisms involved in P dynamics in the soils should be considered carefully.

2. Roles and applications of organic acid transporters

Loss of function analysis and some supportive results indicated that AtALMT3 and AtMATE-PI1 are the malate and citrate transporters induced by P deficiency, respectively. Both of them were located in the root hair cells. Therefore, it is concluded that these transporters are functioned for organic acid transport in the root hair under P deficient condition. It is the first report to characterize the organic acid transporters induced by P deficiency.

The detailed mechanisms of organic acid transport stimulated by P deficiency have not been well explained. Here, it is firstly found that AtALMT3 and AtMATE-PI1 are involved in malate and citrate exudation from roots under P deficient conditions. These molecules could be models to analyze the detailed mechanisms of organic acid transport from roots to the rhizosphere under the P deficiency. The important properties such as substrate specificity and kinetics of transport are not yet clarified in this study. It is required to further analysis to clarify the detail roles of them in the future.

Recently, the mechanism of regulation for TaALMT1, a malate transporter activated by Al stress, was reported by Furuichi et al. (2010). They concluded that an extracellular hydrophilic carboxyl terminal domain regulated the activity of this. It might be the useful knowledge to reveal the function and structure of AtALMT3.

Delhaize et al. (2001) reported that transgenic tobacco plants overexpressing citrate synthase increased the protein but it did not show increased accumulation of citrate in roots or increased Al-activated efflux of citrate from roots. This fact suggests that not only enzymes involved in the metabolism of organic acids but also transporters for exudation of organic acids to the rhizosphere are important for organic acid secretion. AtALMT3 and AtMATE-PI1 have the roles of organic acid transport, therefore, they could be helpful molecules to increase organic acid exudation.

3. Interactions between P deficiency and Al stress on the responses and molecular regulation

In acidic soil, Al stress and P deficiency occur at the same time. Therefore we have to consider the relationships between P and Al stresses. Expression of AtMATE, which confers Al tolerance in *Arabidopsis thaliana* by a mechanism based on Al-activated root citrate exudation, has been found to depend on STOP1 (Liu et al. 2009), a zinc finger transcription factor involved in tolerance to protons in *Arabidopsis thaliana* (Iuchi et al. 2007). In fact, STOP1 appears to be involved in signal transduction pathways regulating a number of Al- and H^+-responsive genes in *Arabidopsis thaliana* (Sawaki et al. 2009), revealing a broader effect that goes beyond that in AtMATE and AtALMT1 (Liu et al. 2009). Yamaji et al. (2009) reported a zinc finger transcription factor ART1 regulates genes for Al tolerance in rice and Tsutsui et al. (2011) identify the cis-acting element as GGN(T/g/a/C)V(C/A/g)S(C/G) by gel-shift assay. On the other hands, PHR1 has been positively identified to mediate P starvation responses (Rubio et al. 2001; Franco-Zorilla et al. 2004). The MYB-like domain of PHR1 binds to a DNA motif GNATATNC, termed P1BS (Rubio et al. 2001), which is present in the promoter of many Pi starvation-induced genes (Franco-Zorilla et al. 2004; Muller et al. 2007). Promoter sequences of *AtALMT3* and *AtMATE-PI1* contained P1BS element, although it could not be detected in the promoter sequence of *AtALMT1*. Promoter sequence of AtALMT1 contained many ART1 binding sequences. Therefore, these P deficient inducible organic acid transporters might be regulated by PHR1, as well as STOP1 and ART1 under Al stresses.

Hoekenga et al. (2006) and Kobayashi et al. (2007) concluded that AtALMT1 is one of the important Al tolerances of Arabidopsis. However, the function of AtALMT1 in P deficient condition has not been studied. In chapter 3, it was shown that P uptake by *atalmt3-1* and *atalmt1* from soil was decreased than in WT (Fig. 3-7). The expression of AtALMT3 was specific in epidermis (Fig. 3-5),

whereas AtALMT1 was specific in endodermis (Kobayashi et al. 2007). It is concluded that AtALMT1 and AtALMT3 have not only functionally but also spatially different properties. It can be hypothesized that AtALMT1 is involved in malate transport from endodermis to apoplast not only Al stress condition but also under P deficient condition, while AtALMT3 is involved in malate transport from epidermal cells to the rhizosphere (Fig. 6-1).

The knowledge of the detailed properties of AtMATE is lacking to discuss the similarity or difference to the AtMATE-PI1 characterized in this study. It is also required to characterize both AtMATEs for understanding the detailed mechanisms of citrate transport from roots to the rhizosphere under Al and/ or P stresses.

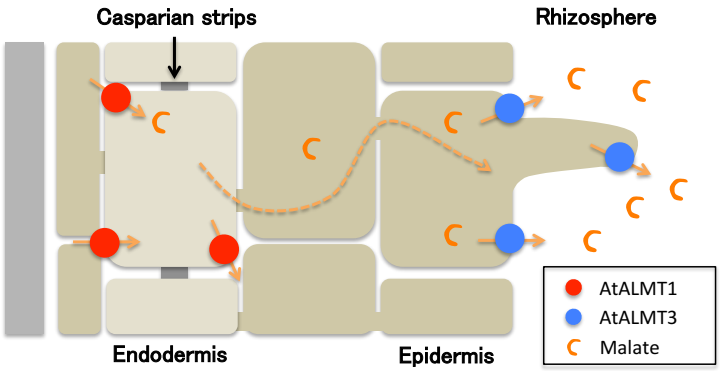

Figure 6-1 Hypothesis of role of AtALMT1 and AtALMT3.

4. Gene modification for utilizing unavailable P in soil

APase activity from Arabidopsis root under –P condition was only 1.3 times higher than that of +P condition (Fig. 2-3). It suggests that Arabidopsis do not have a high ability to increase the secretion of APase induced by P deficient condition, even though Arabidopsis has general ability to secrete APase into the soil. Tadano and Sakai (1991) investigated APase activities of nine crop species, including white lupin grown under +P and –P condition. In this report, APase activity from white lupin roots under –P condition was 19.9 times higher than that of +P condition. That is to say, white lupin has the useful ability to mobilizing P in soil compared with other plants.

In the chapter 5, the effects of exogenous phosphatase and phytase from white lupin, designated LASAP2 and LASAP3, respectively, on plant growth and P uptake were investigated. The positive effects were obtained on the P uptake, although the effects were highly differed by the types of soil. In our recent study, *LASAP2*-overexpressing tobacco has also shown a high ability to use organic P in sterile media (Wasaki et al. 2009). However, utilization of phytate in soil was not very high, although *LASAP2* transformants had somewhat better organic P utilization compared to that of wild type (WT) tobacco. Thus, it is important the solubility of organic P is a limiting factor for mobilization in the soil.

White lupin secretes a huge amount of organic acids and phosphatase from cluster roots under P deficient conditions. It is believed that the exudative burst is the key of the tolerance to P deficiency in white lupin. Uhde-Stone et al. (2005) reported the MATE family protein designated LaMATE, which induced by P deficient lupin roots. However, they could not detect citrate transport activity by this MATE protein. AtMATE-PI1 has only 12% similarity to LaMATE, implying that the property and function are completely different from LaMATE. It is expected to be isolated and characterized an ortholog for AtMATE-PI1 from white lupin, which involved in citrate exudate from P deficient white lupin roots. It is also expected to be isolated and characterized white lupin ortholog for AtALMT3 involved in the malate transport. These are the candidates for application to enhance organic exudations by genetic modifications.

5. Conclusion and perspective

Root-secreted organic acids mobilize sparingly soluble P, not only inorganic but also organic forms. Phosphatase can hydrolyze organic P only after solubilization with organic acids. Therefore, secretion of organic acids is very important for efficient use of not only adsorbed P but also organic P in soil.

This study is the first in the world to report the characterization of malate and citrate transporters induced by P deficiency and involved in secretion to the rhizosphere. It is a helpful knowledge to understand the mechanisms of organic acid transport and to apply the functions for other plants, such as white lupin and/or other main crops. Moreover, it is also found that not only phosphatase but also organic acids are important to mobilize organic P, including phytate. I can conclude that multiple gene modification of organic acid transporters and phosphatases is the key in order to improve the use of unavailable phosphate in soil by gene modification. In the near future, we have to challenge for developing a sustainable agriculture by efficient use of accumulated P in soil. I believe that results shown in this study will be useful for the efficient P utilization in soils.

References

Adams, M.A., and Pate, J.S. (1992). Availability of organic and inorganic forms of phosphorus to lupins (*Lupinus* spp). *Plant and Soil* **145**, 107-113.

Baldwin, J., Karthikeyan, A., and Raghothama, K. (2001). LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. *Plant Physiology* **125**, 728-737.

Baldwin, J.C., Karthikeyan, A.S., Cao, A.Q., and Raghothama, K.G. (2008). Biochemical and molecular analysis of LePS2;1: a phosphate starvation induced protein phosphatase gene from tomato. *Planta* **228**, 273-280.

Cheng, L.Y., Bucciarelli, B., Shen, J.B., Allan, D., and Vance, C.P. (2011). Update on White Lupin Cluster Root Acclimation to Phosphorus Deficiency. *Plant Physiology* **156**, 1025-1032.

Chiu, W.L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996). Engineered GFP as a vital reporter in plants. *Current Biology* **6**, 325-330.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant Journal* **16**, 735-743.

Delhaize, E., Hebb, D.M., and Ryan, P.R. (2001). Expression of a *Pseudomonas aeruginosa* citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux. *Plant Physiology* **125**, 2059-2067.

Delhaize, E., Taylor, P., Hocking, P.J., Simpson, R.J., Ryan, P.R., and Richardson, A.E. (2009). Transgenic barley (*Hordeum vulgare* L.) expressing the wheat aluminium resistance gene (TaALMT1) shows enhanced phosphorus nutrition and grain production when grown on an acid soil. *Plant Biotechnology Journal* **7**, 391-400.

Diener, A.C., Gaxiola, R.A. and Fink, G.R. (2001). Arabidopsis ALF5, a multidrug efflux transporter gene family member, confers resistance to toxins. *Plant Cell* **13**, 1625-1637.

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.

Durrett, T.P., Gassmann, W., and Rogers, E.E. (2007). The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology* **144**, 197-205.

Doolette, A.L., Smernik, R.J., and Dougherty, W.J. (2010). Rapid decomposition of phytate applied to a calcareous soil demonstrated by a solution 31P NMR study. *European Journal of Soil Science* **61**, 563-575.

Fetter, K., Van Wilder, V., Moshelion, M. and Chaumont, F. (2004). Interactions between plasma membrane aquaporins modulate their water channel activity. *Plant Cell* **16**, 215-228.

Franco-Zorrilla, J.M., Gonzalez, E., Bustos, R., Linhares, F., Leyva, A. and Paz-Ares J. (2004). The transcriptional control of plant responses to phosphate limitation. *Journal of Experimental Botany* **55**, 285-293.

Furukawa, J., Yamaji, N., Wang, H., Mitani, N., Murata, Y., Sato, K., Katsuhara, M., Takeda, K., and Ma, J.F. (2007). An aluminum-activated citrate transporter in barley. *Plant and Cell Physiology* **48**, 1081-1091.

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

Gardner, W.K., Barber, D.A., and Parbery, D.G. (1983). The acquisition of phosphorus by Lupinus-albus L. 3. The probable mechanism by which phosphorus movement in the soil root interface is enhanced. *Plant and Soil* **70**, 107-124.

George, T.S., Richardson, A.E., Hadobas, P.A. and Simpson, R.J. (2004) Characterization of transgenic *Trifolium subterraneum* L. which expresses *phyA* and releases extracellular phytase: growth and P nutrition in laboratory media and soil. *Plant, Cell and Environment* **27**, 1351-1361

George, T.S., Simpson, R.J., Hadobas, P.A., and Richardson, A.E. (2005a). Expression of a fungal phytase gene in Nicotiana tabacum improves phosphorus nutrition of plants grown in amended soils. *Plant Biotechnology Journal* **3**, 129-140.

George, T.S., Simpson, R.J., Gregory, P.J., and Richardson, A.E. (2007). Differential interaction of Aspergillus niger and Peniophora lycii phytases with soil particles affects the hydrolysis of inositol phosphates. *Soil Biology and Biochemistry* **39**, 793-803.

George, T.S., Richardson, A.E., Smith, J.B., Hadobas, P.A., and Simpson, R.J. (2005b). Limitations to the potential of transgenic Trifolium subterraneum L. plants that exude phytase when grown in soils with a range of organic P content. *Plant and Soil* 278, 263-274.

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

Green, L.S., and Rogers, E.E. (2004). FRD3 controls iron localization in Arabidopsis. *Plant Physiology* 136, 2523-2531.

Griffiths, B. S., K. Ritz, N. Ebblewhite, and G. Dobson. (1999). Soil microbial community structure. Effects of substrate loading rates. *Soil Biology and Biochemistry* 31, 145-153.

Hammond, J.P., Bennett, M.J., Bowen, H.C., Broadley, M.R., Eastwood, D.C., May, S.T., Rahn, C., Swarup, R., Woolaway, K.E., and White, P.J. (2003). Changes in gene expression in Arabidopsis shoots during phosphate starvation and the potential for developing smart plants. *Plant Physiology* 132, 578-596.

Hammond, J.P. and White, P.J. (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. *Journal of Experimental Botany* 59, 93-109.

Hammond, J.P. and White, P.J. (2011). Sugar Signaling in Root Responses to Low Phosphorus Availability. *Plant Physiology* 156, 1033-1040.

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 Physiology* 126, 1598-1608.

Hoekenga, O.A., Maron, L.G., Pineros, M.A., Cancado, G.M.A., Shaff, J., Kobayashi, Y., Ryan, P.R., Dong, B., Delhaize, E., Sasaki, T., Matsumoto, H., Yamamoto, Y., Koyama, H., and Kochian, L.V. (2006). *AtALMT1*, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 103, 9738-9743.

Hoffland, E. (1992). Quantitative-evaluation of the role of organic-acid exudation in the mobilization of rock phosphate by rape. *Plant and Soil* 140, 279-289.

Hoffland, E., Findenegg, G.R., and Nelemans, J.A. (1989). Solubilization of rock phosphate by rape .2. Local root exudation of organic-acids as a response to p-starvation. *Plant and Soil* **113**, 161-165.

Hur, Y.J., Lee, H.G., Jeon, E.J., Lee, Y.Y., Nam, M.H., Yi, G., Eun, M.Y., Nam, J., Lee, J.H. and Kim, D.H. (2007). A phosphate starvation-induced acid phosphatase from *Oryza sativa*: phosphate regulation and transgenic expression. *Biotechnology Letters* **29**, 829-835.

Hurley, B.A., Tran, H.T., Marty, N.J., Park, J., Snedden, W.A., Mullen, R.T., and Plaxton, W.C. (2010). The Dual-Targeted Purple Acid Phosphatase Isozyme AtPAP26 Is Essential for Efficient Acclimation of Arabidopsis to Nutritional Phosphate Deprivation. *Plant Physiology* **153**, 1112-1122.

Horton, P, Park, K.J., Obayashi, T., Fujita, N., Harada, H., Adams-Collier, C.J. and Nakai, K. (2007) WoLF PSORT: Protein Localization Predictor. *Nucleic Acids Research*, **35**, w585-w587.

Iuchi, S., Koyama, H., Iuchi, A., Kobayashi, Y., Kitabayashi, S., Ikka, T., Hirayama, T., Shinozaki, K., and Kobayashi, M. (2007). Zinc finger protein STOP1 is critical for proton tolerance in Arabidopsis and coregulates a key gene in aluminum tolerance. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 9900-9905.

Jones, D.L. (1998). Organic acids in the rhizosphere - a critical review. *Plant and Soil* **205**, 25-44.

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 Physiology* **130**, 221-233.

Kania, A., Langlade, N., Martinoia, E., and Neumann, G. (2003). Phosphorus deficiency-induced modifications in citrate catabolism and in cytosolic pH as related to citrate exudation in cluster roots of white lupin. *Plant and Soil* **248**, 117-127.

Kaul, S., Koo, H.L., Jenkins, J., Rizzo, M., Rooney, T., Tallon, L.J., Feldblyum, T., Nierman, W., Benito, M.I., Lin, X.Y., Town, C.D., Venter, J.C., Fraser, C.M., Tabata, S., Nakamura, Y., Kaneko, T., Sato, S., Asamizu, E., Kato, T., Kotani, H., Sasamoto, S., Ecker, J.R., Theologis, A., Federspiel, N.A., Palm, C.J., Osborne, B.I., Shinn, P., Conway, A.B., Vysotskaia, V.S., Dewar, K., Conn, L., Lenz, C.A., Kim, C.J., Hansen, N.F., Liu, S.X.,

Buehler, E., Altafi, H., Sakano, H., Dunn, P., Lam, B., Pham, P.K., Chao, Q., Nguyen, M., Yu, G.X., Chen, H.M., Southwick, A., Lee, J.M., Miranda, M., Toriumi, M.J., Davis, R.W., Wambutt, R., Murphy, G., Dusterhoft, A., Stiekema, W., Pohl, T., Entian, K.D., Terryn, N., Volckaert, G., Salanoubat, M., Choisne, N., Rieger, M., Ansorge, W., Unseld, M., Fartmann, B., Valle, G., Artiguenave, F., Weissenbach, J., Quetier, F., Wilson, R.K., de la Bastide, M., Sekhon, M., Huang, E., Spiegel, L., Gnoj, L., Pepin, K., Murray, J., Johnson, D., Habermann, K., Dedhia, N., Parnell, L., Preston, R., Hillier, L., Chen, E., Marra, M., Martienssen, R., McCombie, W.R., Mayer, K., White, O., Bevan, M., Lemcke, K., Creasy, T.H., Bielke, C., Haas, B., Haase, D., Maiti, R., Rudd, S., Peterson, J., Schoof, H., Frishman, D., Morgenstern, B., Zaccaria, P., Ermolaeva, M., Pertea, M., Quackenbush, J., Volfovsky, N., Wu, D.Y., Lowe, T.M., Salzberg, S.L., Mewes, H.W., Rounsley, S., Bush, D., Subramaniam, S., Levin, I., Norris, S., Schmidt, R., Acarkan, A., Bancroft, I., Brennicke, A., Eisen, J.A., Bureau, T., Legault, B.A., Le, Q.H., Agrawal, N., Yu, Z., Copenhaver, G.P., Luo, S., Pikaard, C.S., Preuss, D., Paulsen, I.T., Sussman, M., Britt, A.B., Selinger, D.A., Pandey, R., Mount, D.W., Chandler, V.L., Jorgensen, R.A., Pikaard, C., Juergens, G., Meyerowitz, E.M., Dangl, J., Jones, J.D.G., Chen, M., Chory, J., Somerville, M.C., and Ar Gen, I. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature* **408**, 796-815.

Kihara, T., Wada, T., Suzuki, Y., Hara, T., and Koyama, H. (2003). Alteration of citrate metabolism in cluster roots of white lupin. *Plant and Cell Physiology* **44**, 901-908.

Kobayashi, Y., Hoekenga, O.A., Itoh, H., Nakashima, M., Saito, S., Shaff, J.E., Maron, L.G., Pineros, M.A., Kochian, L.V., and Koyama, H. (2007). Characterization of *AtALMT1* expression in aluminum-inducible malate release and its role for rhizotoxic stress tolerance in arabidopsis. *Plant Physiology* **145**, 843-852.

Kochian, L.V., Hoekenga, O.A., and Pineros, M.A. (2004). How do crop plants tolerate acid soils? - Mechanisms of aluminum tolerance and phosphorous efficiency. *Annual Review of Plant Biology* **55**, 459-493.

Kovermann, P., Meyer, S., Hortensteiner, S., Picco, C., Scholz-Starke, J., Ravera, S., Lee, Y., and Martinoia, E. (2007). The Arabidopsis vacuolar malate channel is a member of the ALMT family. *Plant Journal* **52**, 1169-1180.

Kuang, R., Chan, K.H., Yeung, E. and Lim, B.L. (2009). Molecular and Biochemical Characterization of AtPAP15, a Purple Acid Phosphatase with Phytase Activity, in Arabidopsis. *Plant Physiology* **151**, 199-209.

Lan, M., Comerford, N.B., and Fox, T.R. (1995). Organic-anions effect on phosphorus release from spodic horizons. *Soil Science Society of America Journal* **59**, 1745-1749.

Li, D.P., Zhu, H.F., Liu, K.F., Liu, X., Leggewie, G., Udvardi, M. and Wang D.W. (2002). Purple acid phosphatases of *Arabidopsis thaliana* – comparative analysis and differential regulation by phosphate deprivation. *Journal of Biological Chemistry* **277**, 27772–27781.

Li, H., Huang, G., Meng, Q., Ma, L., Yuan, L., Wang, F., Zhang, W., Cui, Z., Shen, J., Chen, X., Jiang, R. and Zhang, F. (2011). Integrated soil and plant phosphorus management for crop and environment in China. A review. *Plant and Soil* **349**, 157-167.

Li, G., Yang, S., Li, M., Qiao, Y. and Wang, J. (2009). Functional analysis of an *Aspergillus ficuum* phytase gene in *Saccharomyces cerevisiae* and its root-specific, secretory expression in transgenic soybean plants. *Biotechnology Letters* **31**, 1297-1303.

Liu, J.F., Wang, X.F., Li, Q.L., Li, X., Zhang, G.Y., Li, M.G., and Ma, Z.Y. (2011). Biolistic transformation of cotton (*Gossypium hirsutum* L.) with the *phyA* gene from *Aspergillus ficuum*. *Plant Cell Tissue and Organ Culture* **106**, 207-214.

Liu, J.P., Magalhaes, J.V., Shaff, J., and Kochian, L.V. (2009). Aluminum-activated citrate and malate transporters from the MATE and ALMT families function independently to confer Arabidopsis aluminum tolerance. *Plant Journal* **57**, 389-399.

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.

López-Bucio, J., de la Vega, O.M., Guevara-Garcia, A. and Herrera-Estrella, L. (2000). Enhanced phosphorus uptake in transgenic to- bacco plants that overproduce citrate. *Nature Biotechnology* **18**, 450–453.

Lung, S.C., Chan, W.L., Yip, W., Wang, L., Yeung, E.C. and Lim, B.L. (2005). Secretion of beta-propeller phytase from tobacco and Arabidopsis roots enhances phosphorus utilization. *Plant Science* **169**, 341–349.

Lung, S.C., Leung, A., Kuang, R., Wang, Y., Leung, P., and Lim, B.L. (2008). Phytase activity in tobacco (*Nicotiana tabacum*) root exudates is exhibited by a purple acid phosphatase. *Phytochemistry* **69**, 365-373.

Ma, J.F. (2005). Plant root responses to three abundant soil minerals: Silicon, aluminum and iron. *Critical Reviews in Plant Sciences* **24**, 267-281.

Ma, J.F., Ryan, P.R., and Delhaize, E. (2001). Aluminium tolerance in plants and the complexing role of organic acids. *Trends in Plant Science* **6**, 273-278.

Magalhaes, J.V., Liu, J., Guimaraes, C.T., Lana, U.G.P., Alves, V.M.C., Wang, Y.H., Schaffert, R.E., Hoekenga, O.A., Pineros, M.A., Shaff, J.E., Klein, P.E., Carneiro, N.P., Coelho, C.M., Trick, H.N., and Kochian, L.V. (2007). A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. *Nature Genetics* **39**, 1156-1161.

Maron, L.G., Pineros, M.A., Guimaraes, C.T., Magalhaes, J.V., Pleiman, J.K., Mao, C.Z., Shaff, J., Belicuas, S.N.J., and Kochian, L.V. (2010). Two functionally distinct members of the MATE (multi-drug and toxic compound extrusion) family of transporters potentially underlie two major aluminum tolerance QTLs in maize. *Plant Journal* **61**, 728-740.

Marschner, P., Neumann, G., Kania, A., Weisskopf, L. and Lieberei, R. (2002) Spatial and temporal dynamics of bacterial community composition in the rhizosphere of cluster roots of white lupin (*Lupinus albus* L.). *Plant and Soil* **246**, 167-174.

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

Mudge, S.R., Smith, F.W., and Richardson, A.E. (2003). Root-specific and phosphate-regulated expression of phytase under the control of a phosphate transporter promoter enables Arabidopsis to grow on phytate as a sole P source. *Plant Science* **165**, 871-878.

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 Journal* **31**, 341-353.

Muller, R., Morant, M., Jarmer, H., Nilsson, L. and Nielsen, T.H. (2007). Genome-wide analysis of the Arabidopsis leaf transcriptome reveals interaction of phosphate and sugar metabolism. *Plant Physiology* **143**,156-171.

Murphy, J., and Riley, J.P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta* **27**, 31-36

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of Bioscience and Bioengineering* **104**, 34-41.

Narang, R.A., and Altmann, T. (2001). Phosphate acquisition heterosis in Arabidopsis thaliana: a morphological and physiological analysis. *Plant and Soil* **234**, 91-97.

Narang, R.A., Bruene, A., and Altmann, T. (2000). Analysis of phosphate acquisition efficiency in different arabidopsis accessions. *Plant Physiology* **124**, 1786-1799.

Nawrath, C., Heck, S., Parinthawong, N. and Metraux, J.P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. *Plant Cell* **14**, 275-286.

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

Neumann, G., and Martinoia, E. (2002). Cluster roots - an underground adaptation for survival in extreme environments. *Trends in Plant Science* **7**, 162-167.

Neumann, G., Massonneau, A., Martinoia, E., and Romheld, V. (1999). Physiological adaptations to phosphorus deficiency during proteoid root development in white lupin. *Planta* **208**, 373-382.

Neumann, G., Massonneau, A., Langlade, N., Dinkelaker, B., Hengeler, C., Romheld, V., and Martinoia, E. (2000). Physiological aspects of cluster root function and development in phosphorus-deficient white lupin (*Lupinus albus* L.). *Annals of Botany* **85**, 909-919.

Nilsson, L., Muller, R., and Nielsen, T.H. (2007). Increased expression of the MYB-related transcription factor, PHR1, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. *Plant, Cell and Environment* **30**, 1499-1512.

Ohwaki, Y., and H. Hirata. (1992). Differences in carboxylic acid exudation among P-starved leguminous crops in relation to carboxylic acid contents in plant tissues and phospholipid level in roots. *Soil Science and Plant Nutrition*. **38**, 235-243.

Okazaki, K., Shinano, T., Oka, N., and Takebe, M. (2010). Metabolite profiling of *Raphanus sativus* L. to evaluate the effects of manure amendment. *Soil Science and Plant Nutrition* **56**, 591-600.

Okazaki, K., Oka, N., Shinano, T., Osaki, M., and Takebe, M. (2008). Differences in the metabolite profiles of spinach (*Spinacia oleracea* L.) leaf in different concentrations of nitrate in the culture solution. *Plant and Cell Physiology* **49**, 170-177.

Omote, H., Hiasa, M., Matsumoto, T., Otsuka, M. and Moriyama, Y. (2006). The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. *Trends in Pharmacological Sciences* **27** 587-593.

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

Palmiter, R.D. (1974). Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undergraded polysomes and messenger ribonucleic acid. *Biochemistry* **13**, 3606–3615.

Park, S.W., Park, Y.G., Jo, J.K. and Chung, W.I. (1994). Stable expression of yeast acid phosphatase in transgenic *Nicotiana tabacum* plants. *Korean Journal of Genetics* **16**, 137-146.

Perrière, G. and Gouy, M. (1996) WWW-Query: An on-line retrieval system for biological sequence banks. *Biochemistry* **78**, 364-369.

Raghothama, K., and Karthikeyan, A. (2005). Phosphate acquisition. *Plant and Soil* **274**, 37-49.

Raghothama, K.G. (1999). Phosphate acquisition. *Annual Review of Plant Physiology and Plant Molecular Biology* **50**, 665-693.

Rengel, Z. (2002). Genetic control of root exudation. *Plant and Soil* **245**, 59-70.

Richardson, A.E., Hadobas, P.A., and Hayes, J.E. (2000). Acid phosphomonoesterase and phytase activities of wheat (*Triticum aestivum* L.) roots and utilization of organic phosphorus substrates by seedlings grown in sterile culture. *Plant, Cell and Environment* **23**, 397-405.

Richardson, A.E., Hadobas, P.A., and Hayes, J.E. (2001). Extracellular secretion of Aspergillus phytase from Arabidopsis roots enables plants to obtain phosphorus from phytate. *Plant Journal* **25**, 641-649.

Richardson, A.E., Hocking, P.J., Simpson, R.J., and George, T.S. (2009). Plant mechanisms to optimise access to soil phosphorus, *Crop Pasture Science* **60**,124–143.

Richardson, A.E. and Simpson, R.J. (2011). Soil Microorganisms Mediating Phosphorus Availability Update on Microbial Phosphorus. *Plant Physiology* **156**, 989-996.

Rovira, A. D. (1969). Plant root exudates. *Botanical Review* **35**, 35-59.

Rogers, E.E., and Guerinot, M.L. (2002). FRD3, a member of the multidrug and toxin efflux family, controls iron deficiency responses in Arabidopsis. *Plant Cell* **14**, 1787-1799.

Rogers, S.O., Bendish A.J. (1985). Extraction of DNA from milligram amounts of fresh herbarium and minifield plant tissues. *Plant Molecular Biology* **5**, 69–76.

Roschzttardtz, H., Seguela-Arnaud, M., Briat, J.F., Vert, G., and Curie, C. (2011). The FRD3 Citrate Effluxer Promotes Iron Nutrition between Symplastically Disconnected Tissues throughout Arabidopsis Development. *Plant Cell* **23**, 2725-2737.

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 and Development* **15**, 2122-2133.

Ryan, P.R., Delhaize, E., and Jones, D.L. (2001). Function and mechanism of organic anion exudation from plant roots. *Annual Review of Plant Physiology and Plant Molecular Biology* **52**, 527-560.

Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuhara, M., Ahn, S.J., Ryan, P.R., Delhaize, E., and Matsumoto, H. (2004). A wheat gene encoding an aluminum-activated malate transporter. *Plant Journal* **37**, 645-653.

Sasaki, T., Mori, I.C., Furuichi, T., Munemasa, S., Toyooka, K., Matsuoka, K., Murata, Y., and Yamamoto, Y. (2010). Closing Plant Stomata Requires a Homolog of an Aluminum-Activated Malate Transporter. *Plant and Cell Physiology* **51**, 354-365.

Sawaki, Y., Iuchi, S., Kobayashi, Y., Ikka, T., Sakurai, N., Fujita, M., Shinozaki, K., Shibata, D., Kobayashi, M., and Koyama, H. (2009). STOP1 Regulates Multiple Genes That Protect Arabidopsis from Proton and Aluminum Toxicities. *Plant Physiology* **150**, 281-294.

Schunmann, P.H.D., Richardson, A.E., Smith, F.W. and Delhaize, E. (2004). Characterization of promoter expression patterns derived from the Pht1 phosphate transporter genes of barley (*Hordeum vulgare* L.). *Journal of Experimental Botany* **55**, 855–865.

Sekiya, K. (1970). Phosphoric acid (in Japanese). In. Ishizawa S (ed) Analysis methods for measuring soil fertility. Pp. 251–253. Yokendo co. Ltd., Tokyo.

Shen, H., He, L.F., Sasaki, T., Yamamoto, Y., Zheng, S.J., Ligaba, A., Yan, X.L., Ahn, S.J., Yamaguchi, M., Sasakawa, H., and Matsumoto, H. (2005). Citrate secretion coupled with the modulation of soybean root tip under aluminum stress. Up-regulation of transcription, translation, and threonine-oriented phosphorylation of plasma membrane H+-ATPase. *Plant Physiology* **139**, 557-557.

Skene, K.R. (2003). The evolution of physiology and development in the cluster root: teaching an old dog new tricks? *Plant and Soil* **248**, 21-30.

Steen, I. (1998). Phosphorus availability in the 21st century. *Phosphorus and Potassium* **217**, 25-31.

Suzuki, K., Okazaki, K., Tawaraya, K., Osaki, M., and Shinano, T. (2009). Gas chromatography-mass spectrometry associated global analysis of rice root exudates under aseptical conditions. *Soil Science and Plant Nutrition* **55**, 505-513.

Tadano, T., Komatsu, K. (1994) Utilization of organic phosphorus in soil by plant roots. *Trans in 15th Word Congress Soil Science* **9**, 521–522.

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.

Tawaraya, K., Horie, R., Saito, A., Shinano, T., Wagatsuma, T., Saito, K. and Oikawa, A. (in press). Metabolite profiling of shoot extracts, root extracts, and root exudates of rice plant under phosphorus deficiency. *Journal of Plant Nutrition.*

Tesfaye, M., Temple, S.J., Allan, D.L., Vance, C.P., and Samac, D.A. (2001). Overexpression of malate dehydrogenase in transgenic alfalfa enhances organic acid synthesis and confers tolerance to aluminum. *Plant Physiology* **127**, 1836-1844.

Thibaud, M.C., Arrighi, J.F., Bayle, V., Chiarenza, S., Creff, A., Bustos, R., Paz-Ares, J., Poirier, Y., and Nussaume, L. (2010). Dissection of local and systemic transcriptional responses to phosphate starvation in Arabidopsis. *Plant Journal* **64**, 775-789.

Tian, L., Peel, G., Lei, Z., Aziz, N., Dai, X., He, J., Watson, B., Zhao, P., Sumner, L., and Dixon, R. (2009). Transcript and proteomic analysis of developing white lupin (*Lupinus albus* L.) roots. *BMC Plant Biology* **9**, 1.

Tomasi, N., Kretzschmar, T., Espen, L., Weisskopf, L., Fuglsang, A.T., Palmgren, M.G., Neumann, G., Varanini, Z., Pinton, R., Martinoia, E., and Cesco, S. (2009). Plasma membrane H+-ATPase-dependent citrate exudation from cluster roots of phosphate-deficient white lupin. *Plant, Cell and Environment* **32**, 465-475.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*. **22**, 4673-4680.

Truog, E. (1930). The determination of the readily available phosphorus in soils. *Journal of the American Society of Agronomy*, **22**, 874–882.

Tran, H.T., and Plaxton, W.C. (2008). Proteomic analysis of alterations in the secretome of *Arabidopsis thaliana* suspension cells subjected to nutritional phosphate deficiency. *Proteomics* **8**, 4317-4326.

Tran, H.T., Qian, W.Q., Hurley, B.A., She, Y.M., Wang, D.W., and Plaxton, W.C. (2010). Biochemical and molecular characterization of AtPAP12 and AtPAP26: the predominant purple acid phosphatase isozymes secreted by phosphate-starved *Arabidopsis thaliana*. *Plant, Cell and Environment* **33**, 1789-1803.

Tsutsui, T., Yamaji, N., and Ma, J.F. (2011). Identification of a Cis-Acting Element of ART1, a C2H2-Type Zinc-Finger Transcription Factor for Aluminum Tolerance in Rice. *Plant Physiology* **156**, 925-931.

Turner, B.L. (2008). Resource partitioning for soil phosphorus: a hypothesis. *Journal of Ecology* **96**, 698-702.

Turner, B.L., and Newman, S. (2005). Phosphorus cycling in wetland soils: The importance of phosphate diesters. *Journal of Environmental Quality* **34**, 1921-1929.

Uhde-Stone, C., Gilbert, G., Johnson, J.M.F., Litjens, R., Zinn, K.E., Temple, S.J., Vance, C.P., and Allan, D.L. (2003). Acclimation of white lupin to phosphorus deficiency involves enhanced expression of genes related to organic acid metabolism. *Plant and Soil* **248**, 99-116.

Uhde-Stone, C., Liu, J., Zinn, K.E., Allan, D.L. and Vance, C.P. (2005). Transgenic proteoid roots of white lupin: a vehicle for characterizing and silencing root genes involved in adaptation to P stress. *Plant Journal* **44** 840-853.

Vance, C., Uhde-Stone, C., and Allan, D. (2003). Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. *New Phytologist* **157**, 423-447.

Wang, L., Li, Z., Qian, W., Guo, W., Gao, X., Huang, L., Wang, H., Zhu, H., Wu, J.W., Wang, D. and Liu, D. (2011). The Arabidopsis Purple Acid Phosphatase AtPAP10 Is Predominantly Associated with the Root Surface and Plays an Important Role in Plant Tolerance to Phosphate. *Plant Physiology* **157**, 1283-1299.

Wang, X., Tang, C., Guppy, C., and Sale, P. (2008). Phosphorus acquisition characteristics of cotton (*Gossypium hirsutum* L.), wheat (*Triticum aestivum* L.) and white lupin (*Lupinus albus* L.) under P deficient conditions. *Plant and Soil* **312**, 117-128.

Wang, X.R., Wang, Y.X., Tian, J., Lim, B.L., Yan, X.L., and Liao, H. (2009). Overexpressing AtPAP15 Enhances Phosphorus Efficiency in Soybean. *Plant Physiology* **151**, 233-240.

Wasaki, J., Yamamura, T., Shinano, T., and Osaki, M. (2003). Secreted acid phosphatase is expressed in cluster roots of lupin in response to phosphorus deficiency. *Plant and Soil* **248**, 129-136.

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

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

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

Wasaki, J., Rothe, A., Kania, A., Neumann, G., Römheld, V., Shinano, T., Osaki, M., and Kandeler, E. (2005) Root exudation, phosphorus acquisition, and microbial diversity in the rhizosphere of white lupine as affected by phosphorus supply and atmospheric carbon dioxide concentration. *Journal of Environmental Quality* **34**, 2157-2166.

Wasaki, J., Maruyama, H., Tanaka, M., Yamamura, T., Dateki, H., Shinano, T., Ito, S., and Osaki, M. (2009). Overexpression of the LASAP2 gene for secretory acid phosphatase in white lupin improves the phosphorus uptake and growth of tobacco plants. *Soil Science and Plant Nutrition* **55**, 107-113.

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

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

Williamson, L.C., Ribrioux, S., Fitter, A.H. and Leyser, O. (2001). Phosphate availability regulates root system architecture in Arabidopsis. *Plant Physiology* **126**, 875–882

Won, S.K., Lee, Y.J., Lee, H.Y., Heo, Y.K., Cho, M., and Cho, H.T. (2009). cis-Element- and Transcriptome-Based Screening of Root Hair-Specific Genes and Their Functional Characterization in Arabidopsis. *Plant Physiology* **150**, 1459-1473.

Xiao, K., Harrison, M.J., and Wang, Z.Y. (2005). Transgenic expression of a novel *M. truncatula* phytase gene results in improved acquisition of organic phosphorus by Arabidopsis. *Planta* **222**, 27-36.

Xiao, K., Katagi, H., Harrison, M., and Wang, Z.Y. (2006). Improved phosphorus acquisition and biomass production in Arabidopsis by transgenic expression of a purple acid phosphatase gene from *M. truncatula*. *Plant Science* **170**, 191-202.

Yamagishi, M.Y.M., Zhou, K.Q., Osaki, M., Miller, S.S., and Vance, C.P. (2011). Real-time RT-PCR profiling of transcription factors including 34 MYBs and signaling components in white lupin reveals their P status dependent and organ-specific expression. *Plant and Soil* **342**, 481-493.

Yan, F., Zhu, Y., Müller, C., Zörb, C. and Schubert, S. (2002). Adaptation of H+-pumping and plasma membrane H+ ATPase activity in proteoid roots of white lupin under phosphate deficiency. *Plant Physiology* **129**: 50-63

Yokosho, K., Yamaji, N., Ueno, D., Mitani, N., and Ma, J.F. (2009). OsFRDL1 Is a Citrate Transporter Required for Efficient Translocation of Iron in Rice. *Plant Physiology* **149**, 297-305.

Zhang, W., Gruszewski, H.A., Chevone, B.I. and Nessler, C.L. (2008). An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. *Plant Physiology* **146**, 431-440.

Zhang, W.H., Ryan, P.R., and Tyerman, S.D. (2004). Citrate-permeable channels in the plasma membrane of cluster roots from white lupin. *Plant Physiology* **136**, 3771-3783.

Zhou, K., Yamagishi, M., Osaki, M. and Masuda, K. (2008). Sugar signalling mediates cluster root formation and phosphorus starvation-induced gene expression in white lupin. *Journal of Experimental Botany* **59**, 2749–2756

Zimmermann, P., Zardi, G., Lehmann, M., Zeder, C., Amrhein, N., Frossard, E., and Bucher, M. (2003). Engineering the root-soil interface via targeted expression of a synthetic phytase gene in trichoblasts. *Plant Biotechnology Journal* **1**, 353-360.

Acknowledgment

本研究の遂行および本論文の作成に当たり、懇切なご指導を賜りかつ主査として本論文の審査に当たっていただきました、広島大学大学院生物圏科学研究科の和崎淳准教授に謹んで感謝申し上げます。広島大学大学院生物圏科学研究科の河野憲治教授、櫻井直樹教授、実岡寛文教授にはご多忙にも関わらず本論文のご校閲を戴き、ならびに副査として本論文の審査に当たっていただきましたこと、篤く御礼申し上げます。

本研究の実施にあたっては、岡山大学資源植物科学研究所の山本洋子教授ならびに佐々木孝行助教には共同研究として様々な研究手法のご指導やアドバイスを戴きました。北海道農業試験場の信濃卓郎教授ならびに岡崎圭毅さんにはメタボローム関係の分析や解析において大変お世話になりました。謹んで感謝の意を表します。広島大学大学院総合科学研究科の久我ゆかり教授には共同ゼミにおいて的確なご指摘を多々戴きました。広島大学総合科学研究科の浮穴和義准教授にはリアルタイム PCR 装置を、広島大学総合科学研究科の椋田崇生助教には蛍光顕微鏡を快く使用させていただき、実験をスムーズに進めることができました。深く感謝の意を表します。また、東北大学大学院農学院の小島創一助教、北海道大学大学院農学院の大崎満教授ならびに渡部敏裕助教、山形大学の我妻忠雄名誉教授ならびに俵谷圭太郎教授には学会等でお世話になり、研究について様々な助言を戴きました。心より感謝申し上げます。

日々の研究室生活においては、同研究室にて共に研究を行った卒業生の中野陽介君、上脇葉子さん、古谷あゆ美さん、水上奈緒子さん、修士生の矢倉興士君、松崎弘佑君、学部生の入江智史君、津村暁彦君のおかげで日々楽しく、充実した研究室生活を送ることができました。その他にも多くの方々に御支援を賜りました。すべての皆様に深く御礼申し上げます。

最後に、遠方での研究生活を長い間様々な面で支えていただいた両親、姉、弟、ならびに親族一同に心から感謝致します。

2012 年 1 月 12 日　丸山 隼人

Summary

1　緒論

リン肥料の原料であるリン鉱石は地球規模で枯渇が憂慮されている。その一方で、畑などの土壌中には施肥したリンが難溶性リンや有機態リンといった作物が直接利用できないリン（難利用性リン）として残存している。そのため、作物に土壌中の難利用性リンを効率的に吸収させることは、農学上重要な課題の一つである。植物は根から有機酸や酸性ホスファターゼを分泌して土壌中難利用性リンを可給化すると考えられているが、詳細な機構については未解明の部分が多く、特にリン欠乏条件での有機酸の分泌を担う分子はこれまでに知られていない。そこで本研究は、モデル植物であるシロイヌナズナと低リン耐性の強いシロバナルーピンの土壌中の難利用性リン可給化機構を明らかにすることを目的として、以下の４つの実験を実施した。

2　シロイヌナズナの低リン応答

モデル植物のシロイヌナズナはゲノムが解読され、リン欠乏での体内代謝変動や遺伝子発現の網羅的な解析が実施されている。しかしながら、リン欠乏での詳細なリン可給化機構は明らかになっていない。そこで、本章ではシロイヌナズナのリン可給化能力を評価することを目的とし、根分泌物の解析を行った。

リン栄養条件の異なる条件で栽培したシロイヌナズナ根を材料とし、根分泌物中の低分子代謝産物を GC-MS を用いて網羅的に解析した。その結果、リン欠乏条件ではリンゴ酸、フマル酸、クエン酸などの有機酸や、アルギニン、スレオニン、グルタミン酸などのアミノ酸、スクロース、アラビノースなどの糖の分泌が高まることが示された。根分泌液中のホスファターゼ活性およびリンゴ酸、クエン酸濃度を定量的に測定したところ、いずれもリン欠乏で有意に増加していた。

以上の結果から、シロイヌナズナはリン欠乏条件で多くの代謝産物を分泌しており、他の一般的な植物と同様に根分泌物を通した普遍的なリン可給化機構を保持していることが示唆された。

3 シロイヌナズナ根におけるリン欠乏誘導型リンゴ酸トランスポーターの同定と機能解析

根から分泌されるリンゴ酸は土壌中からのリン可給化に重要な役割を果たすと考えられている。シロイヌナズナは低リン条件に応答してリンゴ酸の分泌を誘導することが前章で示されたことから、本章ではシロイヌナズナにおけるリン欠乏条件でのリンゴ酸分泌を担うトランスポーターを新規に同定し、その機能解析を実施することを目的とした。

ALMT タンパク質はアルミニウムに応答したリンゴ酸分泌を担うトランスポーターファミリーである。シロイヌナズナのゲノムには *ALMT* 遺伝子が 14 個存在する。これらのうち、シロイヌナズナの根において再現性よくリン欠乏で誘導される ALMT 遺伝子をマイクロアレイ解析および RT-PCR での発現解析によって調査したところ、*AtALMT3* が選抜された。2 系統の T-DNA 挿入変異株を用いて *AtALMT3* 遺伝子変異による形質を調査したところ、1 系統は *AtALMT3* の発現がノックダウンし、根分泌リンゴ酸量が減少した。もう 1 つの系統では、*AtALMT3* が過剰発現し、根分泌リンゴ酸量が増加した。また、*AtALMT3* の promoter::GUS コンストラクトを作成して発現の組織局在性を調査したところ、*AtALMT3* は根端分裂組織のすぐ上部に存在する根毛細胞で強く発現していた。AtALMT3::GFP 融合タンパク質を発現させた個体により細胞内局在を調査した結果、GFP の蛍光は細胞膜に認められた。土耕栽培で *AtALMT3* の変異株を栽培したところ、リンゴ酸の分泌が減少した系統はリンの吸収能力が野生株と比べて減少していた。これらのことから、AtALMT3 はリン欠乏に応答して根毛からリンゴ酸を分泌するトランスポーターであり、分泌されたリンゴ酸が土壌中のリン可給化に効果があることが示唆された。

4　シロイヌナズナ根におけるリン欠乏誘導型クエン酸トランスポーターの同定と機能解析

根から分泌されるクエン酸もまた、土壌中のリン可給化に重要であることが知られている。前々章において、シロイヌナズナはリン欠乏に応答してクエン酸の分泌が上昇することが示されたことから、本章ではシロイヌナズナにおけるリン欠乏条件でのクエン酸分泌を担うトランスポーターを新規に同定し、その機能解析を実施することを目的とした。

MATE タイプ遺伝子をマイクロアレイ解析の結果から選抜したところ、根毛特異的に発現すると報告のある 1 つの MATE タイプ遺伝子がリン欠乏条件で誘導されることが示唆された。この遺伝子を *AtMATE-PI1* と名付けて T-DNA 挿入変異株を用いた機能の調査を行

った。２系統のノックアウト変異株では、遺伝子発現と根分泌クエン酸量が野生株と比較してともに減少していた。また、これらの変異株を土耕栽培したところ、野生株と比べてリンの吸収能力が減少していた。これらのことから、AtMATE-PI1 は根毛で特異的に発現し、リン欠乏条件下で根毛からのクエン酸分泌を担うトランスポーターであることが示唆された。

5　シロバナルーピン由来ホスファターゼおよびフィターゼ導入による植物の土壌中有機態リン利用

低リン耐性の強い植物であるシロバナルーピンの有機態リンの獲得に関して、これまでに根分泌性の酸性ホスファターゼ *LASAP2* とそのホモログ遺伝子 *LASAP1*、フィターゼ活性をもつ *LASAP3* が単離されている。しかしながら、これらの遺伝子発現に関する詳細な調査は行われていない。また、これまでに LASAP2 を導入したタバコは土壌中のリンを利用する能力が高まる結果を得ている。しかしながら、土壌中有機態リンの主な形態であるフィチン酸の利用にはフィターゼが重要であるが、LASAP2 はフィチン酸に対して特異性が低い。そこで、この章では、シロバナルーピンの有機態リン利用における LASAP1, 2, 3 の役割を明らかにし、さらにフィターゼ活性をもつ LASAP3 を導入したタバコを新たに作出し、土壌中フィチン酸の利用に対するホスファターゼとフィターゼの効果を検証することを目的とした。

LASAP1, LASAP2, LASAP3 の発現解析を行った結果、*LASAP1* はリン欠乏で誘導はされたが全ての器官で恒常的に発現することが示された。*LASAP2* はこれまでの知見と同じくリン欠乏の根、特にクラスター根で特異的に発現していた。*LASAP3* は根を含む全ての器官で発現したが、特に登熟過程の子実において多く発現していた。これらのホスファターゼのアミノ酸配列をシロイヌナズナゲノムに存在する 29 個の全ホスファターゼと比較したところ、LASAP1 と LASAP2 はシロイヌナズナにおいてもリン欠乏で発現が上昇し、根から分泌すると考えられるグループと近いことが示された。LASAP3 はフィターゼ活性をもつと報告のある AtPAP15 と近かった。これらのことから、リン欠乏条件での土壌中からのリン利用において特に LASAP2 が重要であることが確認された。LASAP3 はフィターゼ活性をもつこと、根においても比較的高い発現が確認されたことから、土壌中のフィチン酸の利用にも関与するものと考えられた。

LASAP2 と LASAP3 を導入したタバコは野生株と比べて根からの分泌ホスファターゼ活性が有意に上昇していた。LASAP2 と LASAP3 を導入した系統をリン施肥区、フィチン

酸施肥区、リン無施肥区を設け、リン吸着能力が異なる 2 種類の土壌で栽培した。その結果、LASAP2 および LASAP3 形質転換系統の乾物重量とリン吸収量は、両方の土壌のすべての処理区で野生株より高かった。このことから、LASAP2 および LASAP3 のようなホスファターゼとフィターゼの遺伝子を植物に導入することは、土壌中のリン利用を改善するための手段として効果があることが実証された。また、リン吸着能力の高い黒ボク土におけるリン利用効率は、リン吸着能力の低いマサ土と比べて顕著に低かった。その一方で、系統間のリン利用効率には有意差は認められなかった。これらの結果から、フィチン酸由来のリン利用のボトルネックは酵素の基質特異性ではなく土の中の可溶性であると考えられた。

6　まとめ

本研究においては、これまで詳細な調査が行われていなかったシロイヌナズナの低リン条件でのリン可給化に関わる根分泌物の特性を明らかにした。また、これまで未解明であったリン欠乏条件でのリンゴ酸ならびにクエン酸の分泌に関わるトランスポーターの同定に成功した。低リン耐性の強いシロバナルーピンでは、LASAP2 は土壌中からの積極的なリン可給化に、LASAP3 は土壌中のフィチン酸可給化にそれぞれ貢献することが示唆された。LASAP2 と LASAP3 の導入系統は野生株と比べてリンの吸収能力が向上した。しかしながら、土壌中の有機態リンが可溶性の形態で存在することが有機態リンの利用においても重要だと考えられた。以上より、植物自身による土壌中難利用性リン可給性を向上するためには、有機酸トランスポーターとホスファターゼの同時組換えが鍵であるという結論を得た。