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Stress Response Versus Stress Tolerance: A Transcriptome Analysis of Two Rice Lines Contrasting in Tolerance to Phosphorus Deficiency

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Abstract Transcriptional profiling has identified genes associated with adaptive responses to phosphorus (P) deficiency; however, distinguishing stress response from tolerance has been difficult. We report gene expression patterns in two rice genotypes (Nipponbare and NIL6-4 which carries a major QTL for P deficiency tolerance (*Pup1*)) grown in soil with/without P fertilizer. We tested the hypotheses that tolerance of NIL6-4 is associated with (1) internal P remobilization/redistribution; (2) enhanced P solubilization and/or acquisition; and (3) root growth modifications that maximize P interception. Genes responding to P supply far exceeded those differing between

genotypes. Genes associated with internal P remobilization/redistribution and soil P solubilization/uptake were stress responsive but often more so in intolerant Nipponbare. However, genes putatively associated with root cell wall loosening and root hair extension (xyloglucan endotransglycosylases/hydrolases and NAD(P)H-dependent oxidoreductase) showed higher expression in roots of tolerant NIL6-4. This was supported by phenotypic data showing higher root biomass and hair length in NIL6-4.

Keywords Phosphorus uptake · Phosphorus efficiency · *Pup1* · Root growth · Root hairs · Cell wall

Raw data files have been submitted to GEO (<http://www.ncbi.nlm.nih.gov/geo/>) and will be publicly available as entry GSE6187—Comparative gene expression analysis between Nipponbare and NIL6-4 under two conditions.

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Introduction

About 5.7 billion hectares worldwide lack sufficient plant-available phosphorus (P) to support high crop productivity (Batjes 1997), and almost 50% of rice (*Oryza sativa* L.) soils are currently considered P deficient. Though this deficiency could be corrected through fertilizer application, many farmers in low-input farming ecosystems are too resource poor or simply do not have access to P fertilizers (Kirk et al. 1998). Furthermore, suitable high-grade rock phosphates represent a finite resource near depletion (Runge-Metzger 1995). Efforts are therefore being made to develop cultivars with improved tolerance to P deficiency (Ismail et al. 2007).

Phosphorus is one of the least mobile nutrients in the soil because it is readily bound by soil particles. Plants typically react to such low P availability with a series of adaptive responses that maximize Pi interception through modifications in root growth, enhance P acquisition efficiency through efficient Pi uptake mechanisms combined with exudation of P solubilizing compounds, and improve internal

P use efficiency as a result of rapid P remobilization and retranslocation. For more detailed reviews, the reader may be referred to recent reviews by Karandashov and Bucher (2005), Raghothama and Karthikeyan (2005), and Lambers et al. (2006).

Microarray studies in *Arabidopsis thaliana* (Hammond et al. 2003; Misson et al. 2005; Morcuende et al. 2007) and rice (Wasaki et al. 2003; Wang et al. 2006; Wasaki et al. 2006) have confirmed the Pi responsiveness of many gene families involved in Pi acquisition and P remobilization and redistribution. Despite the valuable insights gained from these studies, it has so far been difficult to draw practically relevant conclusions because distinguishing stress response mechanisms/genes from those leading to elevated tolerance remains difficult. Furthermore, attempts to improve tolerance to P deficiency through transgenic approaches have so far not yielded great success. Overexpression of the root-specific high-affinity transporter gene *HORvuPht1;1* from barley did not enhance P uptake of transgenic plants grown in soil or in hydroponics although the authors had shown in an initial experiment that *HORvuPht1;1* overexpression increased P uptake in cultured rice cells (Rae et al. 2003, 2004). A phytase gene from *Aspergillus niger* in transgenic *Trifolium subterraneum* improved P uptake of plants supplied with phytate and grown in agar under sterile conditions (Richardson et al. 2001), but this positive effect was not always seen in soil-grown plants (George et al. 2005), possibly because phytate was bound to soil particles and therefore not available as a substrate for phytase.

This very limited success highlights the need to bridge the gap between studies focusing on P response (mostly in nutrient solutions) and more soil or field-based studies with genotypes that do exhibit some degree of tolerance to P deficiency. Considerable genotypic variation in the ability to acquire P under P-deficient field conditions was observed in rice (Fageria and Baligar 1997; Wissuwa and Ae 2001a), and a major QTL for P deficiency tolerance, *Pup1* for *P-uptake 1*, was fine-mapped to a region between 15.1 and 15.6 Mb on chromosome 12 (Wissuwa et al. 2002). Near-isogenic lines (NILs) carrying the *Pup1* allele from tolerant donor parent “Kasalath” consistently showed up to fourfold higher P uptake, dry weight, and grain yield compared to the intolerant recurrent parent “Nipponbare” (Wissuwa and Ae 2001b; Wissuwa 2005). Because P deficiency is a major problem in upland or drought-prone rain-fed lowland (nonirrigated) systems, previous studies (Wissuwa and Ae 2001b; Wissuwa 2005) and the study presented here were conducted under “upland” conditions. While the intolerant donor parent “Nipponbare” is a lowland cultivar, NIL6-4 is near identical; hence, studies under upland conditions characterize the response of the *Pup1* locus in typical drought-prone environments.

To characterize the response to P deficiency of Nipponbare and one of its P deficiency-tolerant NILs (NIL6-4) at the molecular level, we conducted a microarray (Agilent 22 k) study using root and shoot samples from plants grown in soil at two P levels. Annotations of candidate genes at the fine-mapped *Pup1* locus have not revealed any gene related to mechanisms known to directly affect P uptake or P metabolism (Heuer et al. 2009), and only two of the genes present at the *Pup1* locus are present on the Agilent 22 k array. It was therefore not the objective of this study to identify *Pup1* candidate genes but rather to identify tolerance mechanisms and regulatory patterns affected by the *Pup1* locus. In particular, we tested the hypotheses that NIL6-4 is more tolerant to P deficiency due to the differential regulation of genes associated with (1) internal P use efficiency due to more efficient Pi remobilization; (2) enhanced P acquisition through efficient Pi uptake or soil P solubilization; and (3) modifications in root growth that maximize Pi interception. An additional objective was to differentiate stress response from stress tolerance genes.

Results

Phenotype

At 40 days after sowing (DAS), P deficiency had significantly impacted plant growth (Table 1). In the unfertilized (–P) treatment, the average shoot dry weight was 0.69 g per plant compared to 2.57 g per plant in the fertilized (+P) treatment, while average root dry weight was 0.46 and 1.12 g per plant, respectively. That these differences were due to P deficiency was evident from reductions in shoot and root P concentrations of about 50% relative to the fertilized control. Differences between genotypes Nipponbare and NIL6-4 were barely noticeable at 40 DAS and not yet significant, which is in line with observations made in previous experiments (Wissuwa and Ae 2001b). In fact, a sampling time before phenotypic differences became apparent was chosen purposely to ensure that samples were obtained from plants experiencing a similar level of stress. In a second set of plants harvested at 85 DAS, NIL6-4 had more than twice the plant P content and almost twice the shoot dry weight in the –P treatment compared to Nipponbare, whereas genotypic differences were small and mostly nonsignificant in the +P treatment (Table 1). While shoot P concentration of NIL6-4 was significantly higher than Nipponbare at 85 DAS under –P (yet root P concentration was similar), the fact that this was not observed under +P conditions suggests that it is not the result of differing shoot P sink capacities between genotypes.

Table 1 Dry Weight, P Concentration, and Derived Parameters in Shoot and Root Samples Taken from an Experiment Conducted Parallel to the Microarray Experiment

Days	P level	Genotype	Shoot dry weight g plant ⁻¹	Shoot P concentration mg g ⁻¹	Root dry weight g plant ⁻¹	Root P concentration mg g ⁻¹	Plant P content mg plant ⁻¹
40	-P	Nipponbare	0.61	0.77	0.42	0.95	0.86
		NIL6-4	0.76 ns	0.82 ns	0.49 ns	0.90 ns	1.07 ns
	+P	Nipponbare	2.94	1.51	1.18	1.82	6.63
		NIL6-4	2.19 ns	1.70 ns	1.06 ns	1.89 ns	5.72 ns
85	-P	Nipponbare	1.93	0.63	0.74	0.93	1.91
		NIL6-4	3.68**	0.73*	1.33**	0.96 ns	3.96**
	+P	Nipponbare	9.60	1.32	3.43	1.75	18.69
		NIL6-4	12.44 ns	1.28 ns	4.19 ns	1.72 ns	23.25*

Values are means of four replications; plants were grown in soil with (+P) or without (-P) P fertilizer. Microarray samples were taken from similar plants at 40 days after sowing

ns not significant

* $p \leq 0.05$ (significant); ** $p \leq 0.01$ (significant) based on *t* tests between means of genotypes within sampling dates and P levels

Expression profiling of Nipponbare and NIL6-4 with the 22 k Agilent array

A total of 5,851 probes in shoots and 1,263 in roots were significantly regulated by the environmental (E) factor P supply ($p \leq 0.001$, Table 2; the complete list of genes is provided in supplementary Table S1). Of these, the proportion of genes upregulated under P deficiency was roughly equal to the number of downregulated genes in both tissues. A considerable overlap between significantly regulated genes in shoots and roots was detected with 498 of 1,263 (39%) of root genes also detected in shoots (Fig. 1a).

Whereas 27% or 6% of all probe sets present in the array were significantly affected in shoots or roots by the factor environment, less than 2% (272) or 1% (167) were affected in respective tissues by the factor genotype (G) (Table 2). The large majority of significantly regulated genes therefore

showed very similar expression patterns in both genotypes. Where genotypic differences existed, the typical pattern was a downregulation in NIL6-4 relative to Nipponbare. The overlap between both tissues was almost identical for the factor G (38% or 63 of 167 root genes also being detected in shoots) as previously detected for factor E (39%) (Fig. 1b). Among the 63 genes detected in both root and shoot, almost 80% were expressed at a lower level in NIL6-4, suggesting that Nipponbare was more affected by P deficiency in both tissues.

For a small group of genes, significant $G \times E$ interactions were detected (Table 2). Interestingly, the interaction pattern differed considerably between shoots and roots. In shoots, 134 genes (82%) were in the category “-P ↓ NIL6-4 ↑,” indicating that they were generally downregulated under P deficiency but less so in NIL6-4. In roots, the predominant category with 52 genes (63%) was characterized by an upregulation under P deficiency that

Table 2 Number of Genes Significantly ($p \leq 0.001$) Upregulated or Downregulated by Factors Environment (P Supply) and Genotype

Tissue	Factor	Regulation				Total
		-P ↑	-P ↓	NIL6-4 ↑	NIL6-4 ↓	
Shoot	Env	2,883	2,968			5,851
	Genotype			91	181	272
Root	Env	677	586			1,263
	Genotype			42	125	167
	Interaction	-P ↑ NIL6-4 ↑	-P ↓ NIL6-4 ↑	-P ↑ NIL6-4 ↓	-P ↓ NIL6-4 ↓	
Shoot	G × E	12	134	9	8	163
Root	G × E	6	9	52	15	82

Env environment, -P ↑ upregulated by P deficiency, -P ↓ downregulated by P deficiency, NIL6-4 ↑ upregulated in NIL6-4, -P ↓ downregulated in NIL6-4, -P ↑ NIL6-4 ↑ upregulated by P deficiency and more so in NIL 6-4, -P ↓ NIL6-4 ↑ downregulated by P deficiency but less so in NIL 6-4, -P ↑ NIL6-4 ↓ upregulated by P deficiency but less so in NIL 6-4, -P ↓ NIL6-4 ↓ downregulated by P deficiency and more so in NIL 6-4

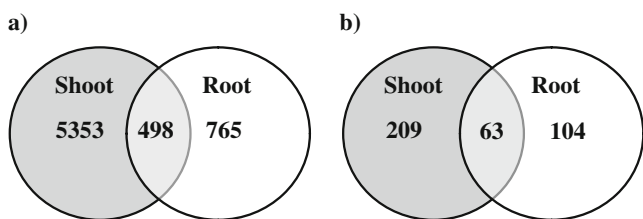


Fig. 1 Number of probes significantly regulated ($p \leq 0.001$) in shoot and root tissue by environment (a) and genotype (b).

occurred to a lesser extent in NIL6-4 ($-P \uparrow$ NIL6-4 \downarrow). Here, too, the more tolerant NIL6-4 appeared less affected by P deficiency compared to Nipponbare, which could reflect a somewhat lower level of stress experienced by the tolerant genotype even before phenotypic differences became significant.

However, this could have been due to (probe) sequence mismatches in NIL6-4 since the Agilent probe set is based on the Nipponbare sequence. An analysis of the chromosomal regions introgressed from Kasalath indicated that 78 of 181 downregulated genes detected in shoots of NIL6-4 were located in introgression regions, while in root, 63 out of 125 genes (50%) were in those regions (supplementary Table S1). We therefore conclude that some artifacts due to

possible probe sequence mismatches affected our results to some degree.

Validation of the microarray

A set of significant genes which are related to P deficiency was selected to validate the microarray data through quantitative polymerase chain reaction (qPCR). They include lipid metabolism (Os02g31030, Os01g04920), phosphatases (Os01g56880, Os01g52230), organic acid (Os02g56310), P transporters (Os04g10750, Os10g30790, AK108907), and cell-wall-related genes (Os11g33270, Os04g51460, Os06g48160). Furthermore, the NAD(P)H-dependent oxidoreductase (Os10g02390) which had the largest fold difference in expression between genotypes was also included in this group. Microarray and qPCR data were in good agreement in terms of separating upregulation and downregulation, even if this opposite pattern was observed in different tissues but of the same probe (Table 3). Some quantitative differences were detected with qPCR data showing considerably higher fold changes for the P transporter OsPT8 (Os10g30790) and the glycerophosphodiesterase (Os02g31030) but slightly lower values for the NAD(P)H-dependent oxidoreductase (Os10g02390).

Table 3 Comparison of Microarray and qPCR Results in Selected Groups of Significant Genes

TIGR5 locus	Shoot				Root			
	MA _s G	MA _s E	qPCR-sG	qPCR-sE	MA _r G	MA-rE	qPCR-rG	qPCR-rE
Os02g31030		<i>3.6</i>		<i>15.4</i>		<i>3.2</i>		<i>1.9</i>
Os01g04920		<i>4.4</i>		<i>3.0</i>		<i>4.1</i>		<i>3.4</i>
Os01g56880		<i>3.3</i>		<i>3.1</i>		<i>3.7</i>		<i>2.4</i>
Os10g30790		<i>2.2</i>		<i>18.4</i>		<i>2.6</i>		<i>1.9</i>
Os01g52230	<i>-1.2</i>	<i>2.8</i>	<i>-1.6</i>	<i>2.3</i>		<i>7.1</i>		<i>1.6</i>
Os04g10750	<i>-1.9</i>		<i>-1.7</i>			<i>2.5</i>		<i>3.4</i>
Os02g56310		<i>2.8</i>		<i>5.1</i>				
Os05g41480		<i>1.7</i>		<i>2.9</i>				
Os04g41680		<i>6.5</i>		<i>2.6</i>				
AK108907		<i>6.1</i>		<i>3.4</i>	<i>-1.7</i>		<i>-1.1</i>	
Os11g33270					<i>2.4</i>		<i>4.5</i>	
Os04g51460					<i>2.3</i>		<i>0.6</i>	
Os06g48160					<i>2.2</i>		<i>3.0</i>	
Os10g02390		<i>0.4</i>		<i>0.5</i>	<i>6.4</i>		<i>4.9</i>	

Data are presented as relative comparison ($-P/+P$) or (NIL6-4/Nipponbare). Data are the means of three independent replications. Effects highlighted in italic print are significant based on two-factorial ANOVAs conducted separately for array and qPCR data at confidence levels $p \leq 0.001$ (array) or $p \leq 0.05$ (qPCR)

MA_sG microarray genotype effect, MA_sE microarray environment effect, Os02g31030 glycerophosphodiester phosphodiesterase, Os01g04920: glycosyl transferase, Os01g56880 purple acid phosphatase, Os10g30790 inorganic phosphate transporter 1–4, Os01g52230 phosphatase phospho1, Os04g10750 inorganic phosphate transporter 1–5, Os02g56310: protein kinase, Os05g41480 plastidic phosphate, Os04g41680 endochitinase A, AK108907 Os high-affinity phosphate transporter 2, Os11g33270, Os04g51460, and Os06g48160 xyloglucan endotransglucosylase/hydrolase, Os10g02390 NAD(P)H-dependent oxidoreductase

Hypothesis 1 NIL6-4 is tolerant to P deficiency due to the differential regulation of genes associated with internal P use efficiency.

Phosphatases

A group of 243 genes annotated as various types of phosphatases (based on TIGR5) was present in the array. P deficiency significantly affected the expression pattern of 88 genes in shoots and 34 genes in roots (Table 4; $p \leq 0.001$). In shoots, just over 50% of phosphatase genes were upregulated under P deficiency whereas about 80% were upregulated in roots, confirming that members of the phosphatase gene family are typically affected by P deficiency (Misson et al. 2005; Morcuende et al. 2007). However, our comparison between genotypes did not reveal a pattern that would suggest that phosphatases were associated with tolerance. In shoots, only four out of 243 phosphatases differed between genotypes with three having higher expression levels in Nipponbare. The one exception was a putative serine/threonine protein phosphatase (Os05g11550) with a 26% higher expression in NIL6-4. In roots, only one gene differed significantly between genotypes. This putative tartrate-resistant acid phosphatase (Os10g02750) was the most P-deficiency-inducible phosphatase in roots with a sevenfold upregulation in both genotypes, albeit at a twofold higher overall level in Nipponbare. P deficiency also affected the expression of several putative ribonucleases, nucleotidases, and adenosine triphosphatases (ATPases) in both tissues, and the more common pattern was a downregulation (Table 4). Genotypic differences were only detected for three root ATPases with Nipponbare having higher expression in all cases (Loc Os03g20949, Os12g28590, and AK071141).

Lipid metabolism

Of the 24 probes putatively annotated as phospholipases, only three showed altered gene expression in response to P deficiency, two being upregulated in shoots and one downregulated in roots (Table 4). Genotype effects were not significant. Alternatively, phospholipid degradation can occur via glycerophosphodiesterases, and one member of this gene family (Os02g31030) was strongly induced by P deficiency in shoots and roots, particularly in NIL6-4 ($G \times E$, $p \leq 0.001$). However, overall, the genotype effect was not significant. In general, it appeared that phospholipid degradation was enhanced, possibly providing sufficient intermediates for subsequent steps in galactolipid and/or sulfolipid synthesis. Two genes coding for enzymes synthesizing the precursor in galactolipid synthesis, UDP-galactose, UDP-glucose-4-epimerase, and UDP-galactose-4-epimerase were induced by P deficiency but in a similar way in both

genotypes. The succeeding steps in galactolipid synthesis facilitated by monogalactosyldiacylglycerol synthase and digalactosyldiacylglycerol synthase were equally induced by P deficiency without differences between genotypes. In contrast to the increase in expression of galactolipid-synthesis-related genes, no change was detected for the second alternative lipid biosynthesis pathway. Sulfolipid-synthesis-related genes UDP-sulfoquinovose synthase (SQD1) and UDP-sulfoquinovosyl:DAG sulfoquinovosyl-transferase (SQD2) were not affected by G or E, which distinguishes our results from those obtained by others for *Arabidopsis* (Misson et al. 2005; Morcuende et al. 2007) or rice (Wang et al. 2006).

Internal Pi transport

A group of genes related to the internal translocation of P including phosphate translocators, glucose-6-phosphate/phosphate translocators (GPT2), ABC transporters, sugar transporters, and antiporters responded to P deficiency, and the response differed between shoots and roots. Transport-related genes were more likely downregulated than upregulated in shoots, but in roots an upregulation was the dominant pattern, particularly for ABC transporters and peptide transporters (Table 4). Genotype effects were only significant for several peptide transporters and only in roots. The putative peptide transporter PTR2 showed a 1.25-fold higher expression (constitutively) in NIL6-4; however, for three other putative PTR2s, Nipponbare showed higher expression at an overall higher level.

Hypothesis 2 NIL6-4 is tolerant to P deficiency due to more efficient Pi uptake mechanisms combined with exudation of P solubilizing compounds.

High-affinity Pi transporters

Of the 13 high-affinity P transporters (OsPT) reported for rice, nine were present on the Agilent array (Table 5). None were localized within Kasalath introgressions of NIL6-4; effects of possible sequence mismatches on expression levels can therefore be ruled out. Most OsPT genes were root specific or at least expressed at a higher level in roots (e.g., OsPT 2, 4, 5, 8, 9, 11). Only OsPT 1 and OsPT 7 showed higher expression in shoots. For those genes with significant G or E effects, the results were similar: gene expression was upregulated under P deficiency, but expression levels were lower in NIL6-4, regardless of P supply (Table 5). However, genotypic differences in gene expression were typically more pronounced in the +P treatment, while the induction by P deficiency was at times stronger in NIL6-4. One exception to the general trend was OsPT12,

Table 4 Gene Families Associated with Internal Remobilization and Redistribution of P Differentially Regulated ($p \leq 0.001$) by P Deficiency (E) and Genotype (G)

Gene family	Significant/total genes	Shoot(G)	Shoot(E)	Root(G)	Root(E)
P scavenging					
Phosphatases	125/243	Os05g11550↑ Os09g15670↓ Os01g43100↓ Os01g58640↓	46↑ 42↓	Os10g02750↓	26↑ 8↓
Ribonuclease	10/27	–	Os09g37006↑ Os11g05570↑ 6↓	–	Os08g33710↑ Os01g67180↑ Os02g52430↓
Nucleotidase	1/3	–	Os01g14810↓	–	–
ATPases	36/134	Os12g24320↓ AK071141↓	15↑ 14↓	Os03g20949↓ Os12g28590↓ AK071141↓	Os01g52560↓ Os03g01120↓ Os03g20949↓
Lipid metabolism					
Phospholipase	3/24	–	Os03g02740↑ Os06g40170↑	–	Os04g35100↓
Glycerophosphodiesterase (GPDE)	1/2	–	Os02g31030↑	–	Os02g31030↑
UDP-glucose 4-epimerase	3/5	–	Os08g28730↑ Os09g15420↑	–	Os09g35800↑
UDP-galactose-4-epimerase	0/2	–	–	–	–
Digalactosyldiacylglycerol synthase (DGD)	1/3	–	Os03g11560↑	–	Os03g11560↑
Monogalactosyldiacylglycerol synthase (MDG)	2/2	–	Os02g55910↑ Os08g20420↑	–	Os02g55910↑ Os08g20420↑
UDP-sulfoquinovose synthase (SQD1)	0/1	–	–	–	–
Sulfoquinovosyldiacylglycerol (SQD2)	0/1	–	–	–	–
Internal P transport					
Phosphate translocators	5/8	–	Os05g41480↑ Os09g12600↑ Os01g07730↓ Os08g25624↓ AK099799↓	–	–
Glucose-6-P translocator	1/2	–	Os08g08840↑	–	–
Miscellaneous transporters					
ABC transporters	8/27	–	Os03g46740↓ Os04g51310↓ Os09g03939↓ Os12g13720↓	–	Os02g11760↑ Os08g43120↑ AK107554↑ Os09g23640↓
Sugar transporters	6/29	–	Os03g36730↑ Os07g10820↑ AK109461↑ Os04g41320↓ Os12g03899↓ AK103844↓	–	–
Peptide transporters	20/52	–	Os05g33960↑ Os11g12740↑ 11↓	Os10g02260↑ Os02g46460↓ Os04g50950↓ Os10g33210↓	Os10g22560↑ Os06g38294↑ Os10g33170↑ Os10g33210↑ Os05g33960↑ Os01g65130↑

Table 4 (continued)

Gene family	Significant/total genes	Shoot(G)	Shoot(E)	Root(G)	Root(E)
					AK100552↓
					Os10g02240↓
					Os10g40600↓
Antiporters	5/16	–	Os11g04030↑ Os12g01580↓ Os04g59600↓ Os10g13940↓	–	Os03g11734↓
MATE	6/16	–	Os10g20450↑ Os03g37470↓ Os10g13940↓		Os03g37640↑ Os03g11734↓ Os10g11860↓
TCA cycle					
Malate dehydrogenase (MDH)	5/11	–	Os10g33800↑ Os01g61380↑ Os03g56280↓ Os07g43700↓	Os07g43700↓	Os07g43700↑ Os08g33720↓
Citrate synthase (CS)	1/6	–	Os02g13840↓	–	–
Aconitase	0/2	–	–	–	–
Isocitrate dehydrogenase (IDH)	2/6	–	Os01g14580↓	–	Os05g49760↓
Succinate dehydrogenase (SDH1)	1/5	–	Os08g02640↓	–	–
Anion transporter	2/5	–	Os03g17740↓ Os04g59550↓	–	–
Central sugar metabolism					
Phosphofructokinase (PFK)	4/9	–	Os01g53680↑ AK069095↑ Os09g24910↑	–	Os05g10650↑
Pyrophosphate: fructose-6-phosphate phosphotransferase (PFP)	4/5	–	Os02g48360↑ Os08g25720↑ AK060464↑ Os06g22060↓	–	–
Phosphoglycerate mutase	3/7	–	Os02g51590↑ Os01g13570↓ Os11g05260↓	–	–
Pyruvate kinase	5/9	–	Os07g08340↑ Os03g46910↑ Os01g47080↑ Os04g58110↑ Os11g05110↑	–	Os07g08340↑ Os03g46910↑ Os01g47080↑
Phosphoenolpyruvate carboxylase (PEPC)	1/7	–	Os01g11054	–	–

Higher expression in NIL6-4 or under P deficiency are denoted by “↑,” opposite patterns by “↓”

which was not expressed in roots and only at very low levels in shoots that decreased further under P deficiency.

Synthesis and exudation of low molecular weight organic acids

Enhanced solubilization of soil-bound P due to root exudation of low molecular weight organic acids

(LMWOAs) is either due to increased LMWOA synthesis, regulated by genes involved in central carbon metabolism and the TCA cycle and/or with enhanced LMWOA efflux from roots. The expression of genes for phosphofructokinase (PFK) and pyruvate kinase was upregulated in P-deficient roots of both genotypes (Table 4), but the key enzyme directing carbon flow to the TCA cycle, phosphoenolpyruvate carboxylase (PEPCase), was not regulated by E

Table 5 Gene Expression Pattern Observed in Shoots and Roots for High-Affinity P Transporters of the OsPT Gene Family

Transporter	GenBank	TIGR locus	Shoot (G)	Shoot (E)	Root (G)	Root (E)
OsPT1	AF536961	Os03g05620	ns	ns	ns	ns
OsPT2	AF536962	Os03g05640	0.536	6.11	<i>0.59</i>	<i>1.12</i>
OsPT3	AF536963	Os10g30770				
OsPT4	AF536964	Os04g10750	0.565	1.61	<i>ns</i>	<i>2.53</i>
OsPT5	AF536965	Os04g10690	0.540	1.72	0.73	2.50
OsPT6	AF536966	Os08g45000				
OsPT7	AF536967	Os03g04360	ns	ns	ns	ns
OsPT8	AF536968	Os10g30790	0.725	2.24	<i>0.80</i>	<i>2.64</i>
OsPT9	AF536969	Os06g21920	ns	ns	<i>0.72</i>	<i>4.14</i>
OsPT10	AF536970	n/a				
OsPT11	AF536971	Os01g46860	ns	ns	<i>0.74</i>	<i>3.67</i>
OsPT12	AF536972	Os03g05610	ns	0.57	nd	nd
OsPT13	AF536973	n/a				

Data are expressed as fold change, with values >1 indicative of upregulation in NIL6-4 or under P deficiency, relative to respective baseline treatment levels Nipponbare or P-fertilized conditions. Italicized values indicate much stronger expression in the respective tissue

or G in roots and neither were pyrophosphate:fructose-6-phosphate phosphotransferase (PFP) and phosphoglycerate mutase (supplementary Table S1). Among TCA-cycle-related genes, an upregulation of citrate synthase (CS) and malate dehydrogenase (MDH) and a downregulation of aconitase are expected to increase cellular levels of the organic acids citrate and malate that are known to effectively solubilize soil P. Only one MDH (Os07g43700; Table 4) showed an upregulation in roots (2.5-fold), but at both P levels the expression was twice as strong in Nipponbare. Citrate synthase and other TCA cycle genes were not affected by factor G and mostly unaffected by E but, if significant, genes were typically downregulated. Putative organic acid efflux transporters of the multidrug and toxin efflux (MATE) or anion transporter gene families were not differentially regulated by factor G in roots (Table 4).

Hypothesis 3 NIL6-4 is tolerant to P deficiency due to modifications in root growth that maximize Pi interception.

Root hairs

Increasing the density or length of root hairs is a very effective way of increasing Pi interception at minimal cost of carbon or P, but very few genes associated with root hair development were found to be differentially regulated in roots (Table 6). Extensins and GPI-anchored proteins were not regulated in roots, and only one out of 34 cellulose synthase-related proteins responded to P deficiency in roots, that gene being downregulated. The cellulose synthase-like D1 (Os10g42750), shown to be required for root hair morphogenesis in rice (Kim et al. 2007), was not affected by E or G. Based on studies with root-hair-defective *Arabidopsis* mutants, several additional genes have been linked to root hair development: guanosine triphosphatase

(GTPase), glycerophosphoryl-diester-phosphodiesterase-like GPI-anchored protein, and the COBRA-like gene COBL9 (Roudier et al. 2002; Hochholdinger et al. 2008). In our study, these genes were not differentially regulated by factors E or G.

However, results of a subsequent independent experiment comparing root hair density and length did suggest the presence of a root-hair-related genotype effect. No difference between Nipponbare and NIL6-4 was observed for root hair density (data not shown), and root hair length did not differ in samples taken from roots grown in a soil that was maintained close to field capacity by daily addition of water. However, in a soil allowed to dry for a 20-day period, root hair length of Nipponbare was reduced by 20–40% whereas this reduction was less severe in NIL6-4 (Fig. 2). Root hairs on first-order lateral roots were longer than on second-order laterals in moist soil, but they were more affected in dry soil, particularly in Nipponbare.

Root growth and cell wall loosening/reassembly

To maintain high relative root growth rates under P deficiency is crucially important and may be directly affected by processes such as cell wall loosening and changes in cell wall composition, which in turn could be mediated by reactive oxygen intermediates (ROI) and plant growth regulators such as auxin. Compared to other potential tolerance mechanisms, we detected a relatively large number of differentially regulated genes for category G in roots. Three members of the xyloglucan endotransglucosylase/hydrolase gene family and one polygalacturonase were expressed at a higher level in NIL6-4, and this pattern was root specific (Table 6). Regulation of ROI-related genes was highly responsive to factor E without showing any obvious pattern. However, for two growth regulators known to affect root growth and architecture,

Table 6 Putative Root Growth-Related Gene Families Significantly Regulated ($p \leq 0.001$) by P Deficiency (E) and Genotype (G)

Gene family	Significant/total genes	Shoot(G)	Shoot(E)	Root(G)	Root(E)
Associated with root hairs and cell wall (loosening)					
GPI-anchored protein	3/10	–	3↑	–	–
Cellulose synthase	8/34	–	3↑ 5↓	–	Os07g36630↓
Extensin	1/8	–	1↓	–	–
Expansin	6/32	–	3↑ Os06g41700↑ 1↓	–	Os06g50960↑ Os06g41700↓
Endoglucanases	8/22	–	4↑ Os06g14540↑ Os08g02220↑ 1↓	–	Os05g33100↑ Os06g14540↓ Os08g02220↓
Xyloglucan endotransglucosylase/hydrolase	9/20	–	6↑ Os06g48160↑ Os11g33270↑	Os04g51460↑ Os06g48160↑ Os11g33270↑	–
Arabinogalactan	4/20	–	3↑ 1↓	–	–
Galactosidase	5/17	–	Os03g06940↑ 2↓	–	Os03g06940↓ Os06g37560↓ Os07g48160↓
Polygalacturonase	9/32	–	7↑ 1↓	Os06g17000↑	Os06g17000↑
Xylanase	0/4	–	–	–	–
Xylanase inhibitor	2/19	–	Os01g71090↑	Os11g47520↓	–
Pectinesterase	7/28	Os10g26680↓	3↑ 2↓	–	Os01g20980↓
Reactive oxygen related					
Peroxidases	39/112	Os03g24380↓	17↑ 11↓	Os05g04490↓	6↑ 7↓
Catalase	2/3	–	–	Os02g02400↑	Os03g03910↑ Os02g02400↓
Glutathione reductase	1/3	–	AK103418↓	–	–
Ascorbate peroxidase	7/10	–	Os03g17690↑ 5↓	–	AK102751↑
Superoxide dismutase	6/6	–	Os05g25850↑ 3↓	–	Os05g25850↑ Os08g44770↑
Dehydroascorbate reductase	2/6	–	2↓	–	–
Unspecific vegetative					
Cell wall protein gp1	2/10	–	Os07g23660↑	Os07g23660↓	Os10g42650↑
Galactosyltransferase	1/9	–	Os07g09670↑	–	–
Laccase	0/2	–	–	–	–
NAD(P)H-dependent oxidoreductase	6/8	2↓	5↓	Os10g02390↑	–
Plant growth-regulator related					
Auxin	34/122	–	18↑ 16↓	Os03g62060↑	Os07g14600↑ Os11g32110↑ AK071997↑
Ethylene	8/46	–	2↑ 4↓	–	Os01g58420↑ Os09g26420↑
Cytokinin	9/37	–	3↑ 3↓	–	Os01g45110↑ Os06g11720↓

Table 6 (continued)

Gene family	Significant/total genes	Shoot(G)	Shoot(E)	Root(G)	Root(E)
Gibberellin	27/62	–	13↑ 7↓	Os07g44910↑	Os11g25454↓ Os02g35940↑ Os05g33730↑ Os07g39470↑ Os11g13570↑ Os06g20200↓ Os10g39140↓ Os12g04150↓

Higher expression in NIL6-4 or under P deficiency are denoted by “↑”, opposite patterns by “↓.” Gene identities are only provided if very few genes per family are significantly regulated or if the same gene is regulated for different tissues or factors

auxin and gibberellin, an increase in transcript abundance was detected in NIL6-4 for one gibberellin receptor (Os07g44910) and a member of the IAA-amino acid hydrolase gene family. This gene (Os03g62060) was the only root-specific member of this family, and P deficiency reduced expression in Nipponbare but not in NIL6-4.

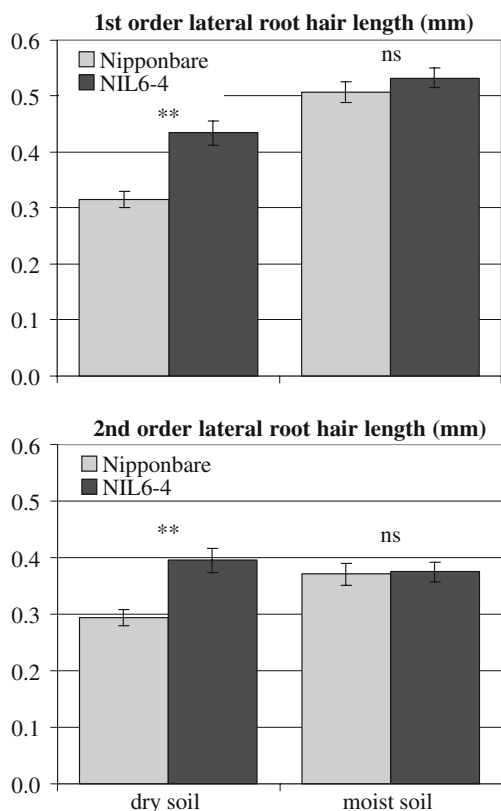


Fig. 2 Root hair length on first- and second-order lateral roots of two genotypes that had been grown for 45 days in 50-L containers filled with a highly P-deficient (P-fixing) soil. The soil was either maintained close to field capacity by daily addition of water or was allowed to dry by withholding water between days 23 and 42. Asterisks denote statistical significance for factor genotype at $p < 0.01$, based on Tukey's HSD (*ns*=not significant).

Transcription factors

Not related to our hypotheses but potentially of high significance for tolerance to P deficiency are regulatory genes. Several stress-responsive transcription factors have been identified with members of the WRKY family being particularly important under P deficiency. Twelve putative WRKYs were differentially regulated in shoots with OsWRKY34 (Os02g43560) showing a pronounced induction under P deficiency (sixfold and threefold in NIL6-4 and Nipponbare, respectively; supplementary Table S1). In contrast, four WRKYs were significantly downregulated in roots, one (Os05g25770) significantly more so in NIL6-4. Transcription factors of the MYB or zinc finger family did not differ between genotypes. Some transcription factors have specifically been associated with the response to P deprivation. They include OsPTF1 (Os06g09370), which has been reported to contribute to tolerance to inorganic phosphate starvation (Yi et al. 2005), the “phosphate-induced protein” (ϕ -1; Os02g52040), and the “phosphate starvation response” factors (PHR1, Os03g21240; PHR2, Os07g25710) that form a regulatory circuit with PHO2 (Os06g40500) and microRNA399 (Fang et al. 2008; Bari et al. 2006). All were only regulated by the factor E and only in shoots.

Differential expression between genotypes: putative stress tolerance genes

Most gene categories investigated so far have shown a much greater response for factor E than for G, corroborating their stress responsiveness but not necessarily their involvement in stress tolerance. Assuming that stress tolerance genes should be differentially expressed between genotypes of high and low tolerance to P deficiency, genes that differed significantly between genotypes were sorted by fold differences for factor G. Among genes with higher expression in shoot tissue of NIL6-4 were an F-box protein, a UDP-glycosyltransferase, an alpha-dioxygenase (located

within the fine-mapped *Pup1* region in chromosome 12), the *O*-methyltransferase ZRP4, and ribosomal proteins (Table 7). In roots, the gene with the biggest positive fold differences in NIL6-4 was an NAD(P)H-dependent oxidoreductase while several other cell-wall-related genes such as a wall-associated protein kinase, three xyloglucan endotransglucosylases (XTH), a xylanase inhibitor, and a polygalacturonase were also more highly expressed in NIL6-4 (Table 8). In addition, two genes showed this expression pattern in both root and shoot, one being a putative prefoldin subunit 2 and the other a dehydroquinase dehydratase/shikimate:NADP oxidoreductase. Most genes with the highest positive fold change in Nipponbare were located within the Kasalath introgressions on chromosomes 1, 7, 10, and 12, and one can therefore not exclude the possibility that sequence mismatches affected the analysis to some extent.

Two additional experiments were carried out to (a) identify a root phenotype that could potentially be linked to the differentially expressed root genes and (b) to independently confirm whether xyloglucan endotransglucosylases and NAD(P)H-dependent oxidoreductase expression patterns cosegregate with *Pup1*. Micrographs from roots of plants grown in well-watered or slightly drought-stressed conditions showed highly developed aerenchyma under both soil water conditions and in both genotypes (supplementary Fig. S1; only Nipponbare is shown). However, genotypic differences were significant for root hair length in drying soil where NIL6-4 maintained longer root hairs compared to Nipponbare (Fig. 2).

In root RNA samples taken from a similar experiment, expression patterns detected for three xyloglucan endotransglucosylases (XTH) and a NAD(P)H-dependent oxidoreductase (see Table 8) were independently confirmed for a set of NILs segregating for the *Pup1* locus (NILs 6-4, Y-4, 14-4 for +*Pup1*; NILs 26-6, 28-10, and Nipponbare for -*Pup1*). The difference between the +/-*Pup1* groups was most pronounced for OsXTH2 followed by OsXTH11 (Fig. 3), and as in the array, +*Pup1* NILs showed constitutively higher expression levels irrespective of P supply. For OsXTH9, the difference between groups was only significant in the +P treatment. We were not able to confirm that the expression of the NAD(P)H-dependent oxidoreductase cosegregated with *Pup1*. Expression of this gene was very variable with highest levels in NILs 6-4 and 28-10 (-*Pup1*).

Discussion

Plant response to P deficiency has been analyzed at the transcriptome level in both *Arabidopsis* (Hammond et al. 2003; Misson et al. 2005; Morcuende et al. 2007) and rice (Wasaki et al. 2003, 2006; Wang et al. 2006). These studies were carried out in nutrient solutions or other artificial

media and had detected a series of adaptive responses at the transcriptome levels that (1) circumvent metabolic steps requiring Pi by utilizing alternative glycolytic or respiratory pathways; (2) alter the lipid composition by enzymatic degradation of phospholipids and synthesis of galactolipid and sulfolipids; (3) remobilize Pi through enhanced expression of phosphatases and RNases; (4) enhance Pi uptake and retranslocation via an upregulation of high-affinity Pi transporters; (5) alter the carbon skeleton flow with potential benefits for root growth or organic acid synthesis and exudation; and (6) are involved in signal transduction and transcriptional regulation. It was generally concluded that the responses in rice and *Arabidopsis* follow similar patterns (Misson et al. 2005; Wang et al. 2006) which was largely confirmed in this study. Despite the insights gained from transcriptomic comparisons of a single genotype under different levels of P supply, it remains difficult to conclude which of the observed changes in gene expression are of practical importance and could therefore be harnessed to improve crop performance under P-limited conditions in the field.

Stress response

Our global analysis revealed that the number of genes affected by the factor E exceeded those differing between genotypes by a factor of seven in roots and a factor of 21 in shoots. Most P-responsive genes could therefore not be associated with the P deficiency tolerance in NIL6-4. For example, we observed upregulation of P stress response genes related to Pi remobilization, including genes involved in lipid metabolism, phosphatases, and other genes belonging to the P scavenging system, as well as genes involved with P transporters, translocators, and antiporters; alteration of carbohydrates, amino acids, and protein metabolism; and alteration of secondary metabolism and photosynthesis. While this confirms other reports that these genes are associated with P stress response in P-deficient plants (Wasaki et al. 2003; Misson et al. 2005; Morcuende et al. 2007), these responses were typically less pronounced in NIL6-4. Our transcriptome data therefore do not support the hypothesis that tolerance of NIL6-4 is the result of superior internal P use efficiency due to more efficient Pi remobilization. This conclusion is supported by experimental evidence showing that shoot P concentrations of Nipponbare were equal to or lower than in *Pup1*-NILs (Wissuwa and Ae 2001b), an observation that we could confirm here (Table 1).

An alternative hypothesis linked tolerance of NIL6-4 to enhanced P acquisition through efficient Pi uptake or soil P solubilization. Our observation that high-affinity P transporters were upregulated under P deficiency is consistent with reports from others in rice (Paszkowski et al. 2002; Ming et al. 2005) and other species (Raghothama and

Table 7 Genes with the Greatest Fold Difference in Shoot Expression Between Genotypes ($p \leq 0.001$; Based on Data from Both P Treatments)

Fold difference	TIGR5-based annotation	TIGR5 locus
Expressed at higher level in NIL6-4		
4.1	F-box domain containing protein	Os10g03620 ^a
3.6	DOGT1 (DON-glucosyltransferase); UDP-glycosyltransferase/transferase, transferring glycosyl groups	AK066178
3.6	Alpha-DOX2, putative	Os12g26290 ^a
2.6	<i>L. esculentum</i> dehydroquinase dehydratase/shikimate:NADP oxidoreductase mRNA, complete cds. PLN	AK058923 ^a
2.5	Prefoldin subunit 2, putative	Os12g30060 ^{a,b}
2.3	<i>O</i> -methyltransferase ZRP4, putative	Os11g20080
2.2	Chloride channel protein CLC-a, putative	Os12g25200
1.9	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, putative	Os12g08170
1.9	Retrotransposon protein, putative, Ty1-copia subclass	Os04g49790
1.9	Aspartate-semialdehyde dehydrogenase, putative	Os03g55280
1.9	Transposon protein, putative, mutator subclass	Os03g55830
1.9	DNA gyrase subunit A, putative	Os03g59750
1.8	40S ribosomal protein SA, putative	Os07g42450
1.8	40S ribosomal protein S6, putative	Os07g42950
1.7	40S ribosomal protein S25-1, putative	Os08g44480
1.7	Phosphoribosylformylglycinamide cyclo-ligase, chloroplast/phosphoribosylaminoimidazole synthetase	AK102151
1.7	5S rRNA binding protein, putative	Os03g41612
1.7	40S ribosomal protein S20, putative	Os03g14530
1.6	RNA binding protein, putative	Os07g23244
1.6	Os ribosomal protein mRNA, complete cds. PLN	AK062150
1.6	Exosome complex exonuclease 1, putative	Os08g02360
1.6	DNA binding protein, putative	Os07g12630
1.6	Glucan endo-1,3-beta-glucosidase 7 precursor, putative	Os02g53200
1.6	Protein TOC75, chloroplast precursor, putative	Os03g16440
Expressed at higher level in Nipponbare		
1.85	Phosphatidylinositol-4-phosphate 5-kinase family protein	Os04g59540
1.85	Inorganic phosphate transporter 1–5, putative	Os04g10750
1.95	Amino acid transporter, putative	Os12g08090 ^a
1.96	Aquaporin TIP1.2, putative	Os01g74450
2.09	F-box domain containing protein	Os10g04700 ^a
2.11	PDI-like protein, putative	Os04g51920
2.14	F-box domain containing protein	Os10g03730 ^a
2.18	Vacuolar protein sorting 26, putative	Os12g31640 ^a
2.21	F-box domain containing protein	Os10g03600 ^a
2.25	Amidohydrolase family protein, putative	Os12g28270 ^a
2.29	dnaJ-like protein, putative	Os01g42190 ^a
2.36	Stripe rust resistance protein Yr10, putative	Os10g04342 ^{a,b}
2.48	Amidohydrolase family protein, putative	Os12g28270 ^{a,b}
2.52	AATP1 (AAA-ATPase 1); ATP binding/ATPase	AK07114 ^{a,b}
2.61	NB-ARC domain containing protein	Os12g10710 ^a
2.69	NAD(P)H-dependent oxidoreductase, putative	Os10g02380 ^{a,*}
2.84	Retrotransposon protein, putative, unclassified	Os12g35460 ^{a,b}
3.10	Nitrilase-associated protein, putative	Os12g31780 ^a
3.20	Disease resistance protein RPM1, putative	Os10g04674 ^{a,b}
3.22	RGH1A, putative	Os12g33160 ^a
3.31	OsWAK98-OsWAK receptor-like cytoplasmic kinase	Os10g02360 ^{a,b}
3.46	csAtPR5, putative	Os12g07500 ^{a,b}
3.49	3- <i>N</i> -Debenzoyl-2-deoxytaxol <i>N</i> -benzoyltransferase, putative	Os12g27254 ^a

Table 7 (continued)

Fold difference	TIGR5-based annotation	TIGR5 locus
4.08	Major myoinositol transporter iolT, putative	Os12g32940 ^a
4.96	Peptidyl-prolyl <i>cis</i> – <i>trans</i> isomerase, putative	Os10g06630 ^{a,b}
5.85	Transposon protein, putative, CACTA, En/Spm subclass	Os10g04050 ^a
5.99	NB-ARC domain containing protein	Os12g32660 ^{a,b}
8.69	Lipoxygenase 2.1, chloroplast precursor, putative	Os12g37260 ^a

Only genes with informative annotation and that are “present” in at least one tissue are shown

^a Localized within Kasalath introgressions present on chromosomes 1, 7, 10, and 12 (Torabi et al. 2009)

^b Genes present in shoot and root

Karthikeyan, 2005). However, we cannot conclude that this observation assigns a critical role to these transporters in stress tolerance because expression levels in our tolerant line NIL6-4 were consistently lower than in Nipponbare. That transporter activity (in addition to transcript level) was indeed not limiting in Nipponbare could be deduced from P depletion experiments showing that Nipponbare is capable of depleting Pi from nutrient solutions as rapidly and to an equally low level (0.16 μ M P) as a *Pup1*-NIL (Wissuwa 2005). Similarly, barley lines overexpressing a Pi transporter did not show improved tolerance to P deficiency (Rae et al. 2004), most likely because substrate (Pi) availability rather than transporter activity is the limiting step in P-deficient soils.

The exudation of LMWOAs capable of releasing soil-bound P is considered to be a highly effective way of increasing the substrate (Pi) availability for uptake (Kirk et al. 1999; Lambers et al. 2006T). P-deficiency-induced alterations in gene expression that would increase carbon supply for organic acid synthesis have been reported for rice (Wasaki et al. 2003) and *Arabidopsis* (Morcuende et al. 2007). We have detected the upregulation of genes, under P deficiency, coding for enzymes related to carbon flow through glycolysis to TCA (such as PFK, PFP, PEPCase, and pyruvate kinase). In addition, P deficiency led to an upregulation of a malate dehydrogenase and a succinate dehydrogenase, but in all cases expression levels did not differ between genotypes. In any case, increased synthesis of LMWOAs is not necessarily indicative of a higher LMWOA exudation since the limiting step is not the plant-internal concentration of LMWOAs (synthesis) but their rate of release (Delhaize et al. 2001). This release is mediated by specific efflux transporters, and their level of activity was shown to be directly linked to malate or citrate-mediated tolerance to excess aluminum (Delhaize et al. 2004; Magalhaes et al. 2007). However, putative efflux transporters were not among the root-specific genes differentially expressed between genotypes, corroborating earlier results that neither genotype exuded citrate or malate

at high enough concentrations to have a significant effect on P solubilization (Wissuwa 2005).

The number of genes regulated in shoots by P stress in our study was higher than in other studies examining P stress response (Wasaki et al. 2006; Misson et al. 2005; Morcuende et al. 2007), likely due to differences in statistical analysis and in stress intensity. Other studies may have used a threshold (e.g., at least twofold higher expression) in their analysis, therefore ignoring genes with smaller changes. Our analysis was based on a mixed-model analysis of variance (ANOVA) using a threshold of $p \leq 0.001$ but no additional fold change threshold. The second potential reason is that our plants were older and had experienced P deficiency for a longer period than the experimental plants used in other studies. They were thus suffering more long-term effects of P deficiency.

Stress tolerance

We can therefore conclude that neither hypothesis 1 nor hypothesis 2 adequately explains observed genotypic differences in tolerance to P deficiency. The question as to which differentially regulated genes could have contributed to tolerance therefore remains unresolved so far and shall now be addressed based on a compilation of the most differentially regulated genes (by fold difference) for factor G. In shoots, no overrepresentation of any specific gene family, metabolic pathway, or functional category was detected that would have suggested the involvement of a specific tolerance strategy (Table 7). However, in roots, cell-wall-related genes were highly overrepresented (Table 8), including three members of the xyloglucan endotransglycosylases hydrolase (XTH) gene family, a xylanase inhibitor, a polygalacturonase, a cell-wall-associated protein kinase, and a root-specific cortical cell-delineating protein (root-specific protein ZRP3, significant for G \times E). In addition, two genes potentially linked to the hormonal regulation of root growth, an IAA-amino acid hydrolase ILR1 precursor and a gibberellin receptor *GID1L2*, were among the genes expressed at a higher level in NIL6-4 roots. The higher expression for many root cell-

Table 8 Genes with the Greatest Fold Difference in Root Expression Between Genotypes ($p \leq 0.001$; Based on Data from Both P Treatments)

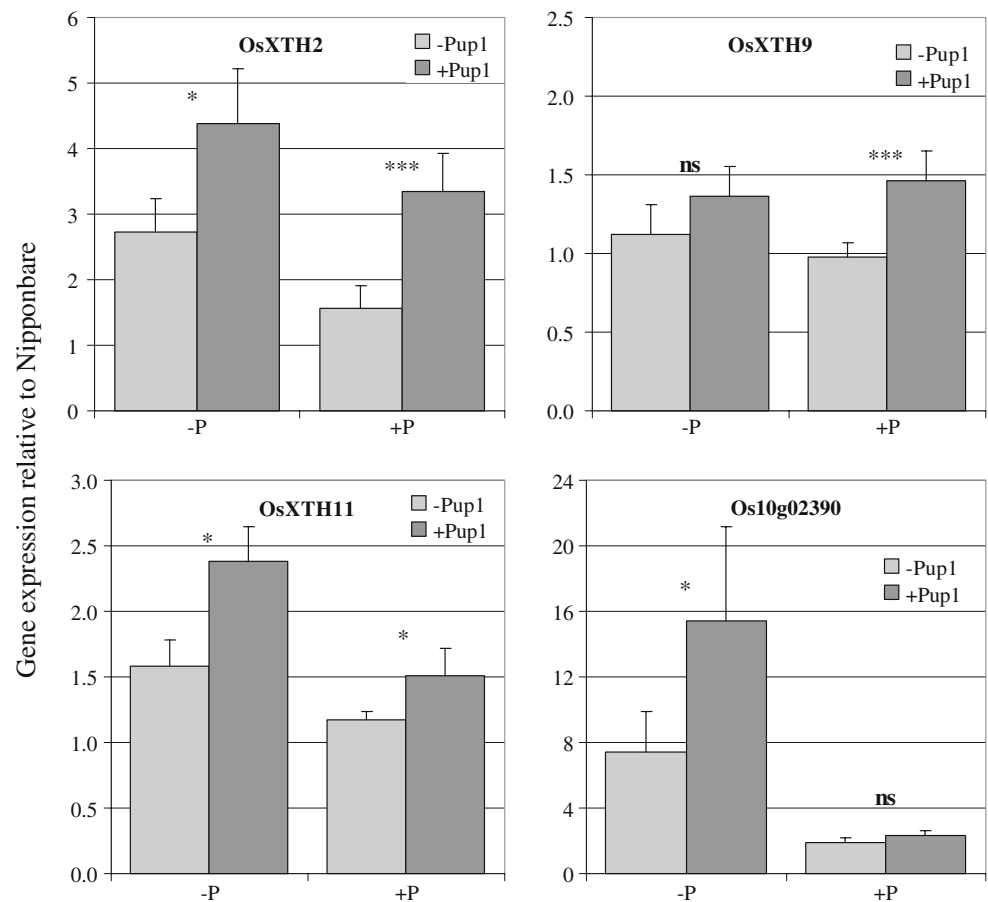
Fold difference	TIGR5-based annotation	TIGR5 locus
Expressed at higher level in NIL6-4		
6.42	NAD(P)H-dependent oxidoreductase, putative	Os10g02390 ^a
2.69	Os wall-associated protein kinase mRNA, complete cds. PLN	AK105147
2.48	Thionin precursor, putative	Os06g32160
2.40	Xyloglucan endotransglucosylase/hydrolase precursor, putative	Os11g33270
2.30	Xyloglucan endotransglucosylase/hydrolase protein 15 precursor, putative	Os04g51460
2.29	Xylanase inhibitor protein 2 precursor, putative	Os11g47520
2.18	Xyloglucan endotransglucosylase/hydrolase protein 23 precursor, putative	Os06g48160
2.11	IAA-amino acid hydrolase ILR1 precursor, putative	Os03g62060
2.06	<i>L. esculentum</i> dehydroquinase dehydratase/shikimate:NADP oxidoreductase mRNA, complete cds. PLN	AK058923 ^b
1.79	Arginine decarboxylase, putative	Os04g01690
1.76	Prefoldin subunit 2, putative	Os12g30060 ^b
1.60	Gibberellin receptor GID1L2, putative	Os07g44910
1.59	Cyclopropane fatty acid synthase, putative	Os12g16650
1.58	ATP-dependent RNA helicase HAS1, putative	Os01g43130
1.51	Polygalacturonase-1 noncatalytic beta subunit precursor, putative	Os06g17000
1.50	Catalase isozyme A, putative	Os02g02400
Expressed at higher level in Nipponbare		
1.68	3- <i>N</i> -Debenzoyl-2-deoxytaxol <i>N</i> -benzoyltransferase, putative	Os07g14080
1.68	Protein kinase domain containing protein	Os11g44560
1.69	Os (japonica cultivar-group) high-affinity phosphate transporter 2 mRNA, complete cds. PLN	AK108907
1.71	Aspartic proteinase nepenthesin-1 precursor, putative	Os10g39260
1.71	Peptide transporter PTR2, putative	Os02g46460
1.75	Triacylglycerol lipase, putative	Os06g10850
1.80	Major pollen allergen Bet v 1-D/H, putative	Os12g36850 ^a
1.92	RGH1A, putative	Os12g36720 ^a
1.98	Vacuolar protein sorting 26, putative	Os12g31640 ^a
2.00	F-box domain containing protein	Os10g04700 ^a
2.02	Tartrate-resistant acid phosphatase type 5 precursor, putative	Os10g02750 ^a
2.10	Cytochrome P450 76C2, putative	Os02g36110
2.12	Retrotransposon, putative, centromere specific	Os10g07160 ^a
2.18	Retrotransposon protein, putative, unclassified	Os12g35460 ^{a,b}
2.20	Bowman–Birk type bran trypsin inhibitor precursor, putative	Os01g03390
2.23	Malate dehydrogenase, glyoxysomal precursor, putative	Os07g43700
2.31	Amidohydrolase family protein, putative	Os12g28270 ^{a,b}
2.46	Transposon protein, putative, unclassified	Os10g05020 ^a
2.48	Amidohydrolase family protein, putative	Os12g28270 ^{a,b}
2.71	Vegetative cell wall protein gp1 precursor, putative	Os07g23640 ^a
3.23	Vegetative cell wall protein gp1 precursor, putative	Os07g23660 ^a
3.95	Peptidyl-prolyl <i>cis</i> – <i>trans</i> isomerase, putative	Os10g06630 ^{a,b}
4.20	F-box domain containing protein	Os10g03600 ^a
5.07	csAtPR5, putative	Os12g07500 ^{a,b}
5.28	NB-ARC domain containing protein	Os12g10330 ^a
5.41	OsWAK98-OsWAK receptor-like cytoplasmic kinase	Os10g02360 ^{a,b}
7.37	Rust resistance-like protein RP1-3, putative	Os12g10340 ^a
8.68	AATP1 (AAA-ATPASE 1); ATP binding/ATPase	AK071141 ^b

Only genes with informative annotation and that are “present” in at least one tissue are shown

^aLocalized within Kasalath introgressions present on chromosomes 1, 7, 10, and 12 (Torabi et al. 2009)

^bGenes present in shoot and root

Fig. 3 Relative gene expression of OsXTH2 (Os11g33270), OsXTH9 (Os06g48160), OsXTH11 (Os06g48160), and NAD(P)H-dependent oxidoreductase (Os10g02390) in a set of NILs segregating for the Pup1 locus. Each data point represents the mean of three NILs (NILs 14-4, 6-4, Y4 for +Pup1; NILs 26-6, 28-10, and Nipponbare for -Pup1), with three biological and two technical replications per NIL. *, **, *** denote significance at the $p < 0.05$, 0.01, and 0.001 levels of significance, respectively (*ns*=nonsignificant).



wall-associated genes would support our third hypothesis that tolerance to P deficiency in NIL6-4 is due to modifications in root growth that maximize Pi interception. This is further corroborated by phenotypic data showing that the most consistent difference between Nipponbare and NIL6-4 is a far more pronounced reduction in root biomass and surface area due to P deficiency in Nipponbare (Wissuwa and Ae 2001b; Wissuwa 2005). Presumably, the increased P uptake by NIL6-4 under P-deficient conditions allowed further root growth compared to Nipponbare: however, under P-replete conditions, both genotypes acquire sufficient P for unrestricted root growth, and hence root biomass did not differ between genotypes.

The XTH genes differentially expressed for G in roots correspond to OsXTH2, OsXTH9, and OsXTH11, which are members of a 15-member XTH gene family in rice (Yokoyama et al. 2004). The role of xyloglucans and XTH in rice remains a matter of debate, but it is thought that they play a role in cell wall restructuring and in cell expansion and therefore in root growth (Yokoyama et al. 2004). OsXTH2 was considered to be root specific which we cannot confirm since it was strongly (fivefold) upregulated by P deficiency in shoots whereas root expression levels were reduced by about 50%, with NIL6-4 showing a constitutively higher expression (2.3 fold) compared to

Nipponbare. An almost identical expression pattern was seen for OsXTH11 while OsXTH9 was constitutively higher in NIL6-4 roots with no difference due to G or E in shoots. A strong upregulation in shoots under stress was also seen for Os07g34580, Os02g46910, and Os03g02610, and in all cases this upregulation was about twice as high in NIL6-4.

Genotypic differences in XTH expression levels under P deficiency could have simply been a consequence of a more pronounced root growth reduction in Nipponbare; however, the observation that XTH's are very strongly upregulated under low P stress in shoots (14-fold for OsXTH11; fivefold for OsXTH2), despite an even stronger biomass reduction in shoots compared to roots (Table 1), contradicts the notion that XTH expression simply follows changes in growth. One may therefore assign a yet unknown function to XTHs under stress. In fact, enhanced expression of XTH has been observed in maize roots under flooding where it was associated with aerenchyma formation (Saab and Sachs 1996) and in drying soil where XTH was thought to help maintain root elongation rates at low water potential (Wu et al. 1994). In addition, the activity of xyloglucan endotransglycosylases has been localized in growing root hairs (Vissenberg et al. 2001).

Aerenchyma formation and root hair elongation are two potential tolerance mechanisms that would allow plants to

produce roots at minimal carbon and Pi cost (Lynch and Ho 2005). In order to determine if Nipponbare had impaired aerenchyma formation, several micrographs of Nipponbare and NIL6-4 roots were taken. It was concluded that Nipponbare developed highly aerenchymatic roots even under fully aerobic soil conditions (supplementary Fig.S1) and that no visible differences existed in aerenchyma development between NIL6-4 and Nipponbare in root samples from both dry and moist soil (data not shown). For root hair length, on the other hand, significant genotypic differences were detected with NIL6-4 having longer hairs in dry soil compared to Nipponbare (Fig. 2). Assuming cylindrical root geometry, the observed difference in hair length between genotypes would result in a 70% greater soil volume explored by the hairy portions of roots of NIL6-4. Based on model simulations by Wissuwa (2003) such an increase might be more than sufficient to account for the differences in P uptake typically detected between Nipponbare and *Pup1*-NILs. This is because any initial small increase in P uptake (ΔP) brought about by longer root hairs would get amplified through a subsequent root growth effect if plants use the ΔP to produce more root biomass, which is typically observed in NIL6-4 (Table 1).

That the difference in root hair length was only seen in drying soil furthermore matches our observation that differences in P uptake between NIL6-4 and Nipponbare increase under low rainfall conditions in field experiments or under imposed intermittent drought in pot experiments (unpublished data). In drying soil, P availability is further reduced, and it has been observed that P becomes the first nutrient-limiting rice growth under drought (Stephan Haeefe, personal communication). That XTH activity was involved in maintaining root elongation rates at low water potential in maize (Wu et al. 1994) could suggest a similar role for rice. Additional indirect evidence suggests a link between root-specific gene expression patterns and genotypic difference in root growth and/or root hair length. Maize root elongation was shown to be dependent on an additional wall-loosening mechanism mediated via ROI, a process initiated by the NAD(P)H oxidase-catalyzed formation of superoxide radicals (O_2^- ; Liskay et al. 2004). One such NAD(P)H-dependent oxidoreductase (Os10g02390) was the gene with the biggest fold change difference between NIL6-4 and Nipponbare (6.4-fold higher in NIL6-4 roots). This was a highly root-specific regulation pattern as expression in shoots at $-P$ was threefold higher in Nipponbare. A link between NAD(P)H-dependent oxidoreductase and root and root hair elongation is supported by data from studies of the root hairless *Arabidopsis* mutant (*rhd2*) which is impaired in the generation of ROIs in the root due to the disruption of a NAD(P)H oxidase gene (Foreman et al. 2003). In subsequent gene expression analyses via reverse transcription (RT)-qPCR, we were able to confirm the high expression of

Os10g02390 in NIL6-4, but all other NILs also showed higher gene expression compared to Nipponbare, raising doubt about the link between the *Pup1* locus and NAD(P)H-dependent oxidoreductase expression.

At this point, knowledge about the regulation of root elongation and root hair growth in graminaceous plants is incomplete with competing views attributing cell wall loosening to either XTH, expansins, or NAD(P)H-oxidoreductase-dependent ROI release (Liskay et al. 2004; Vissenberg et al. 2005). That several genes associated with two of these processes were among the few genes with significantly higher expression in roots of NIL6-4 could therefore suggest that they are involved in the ability to maintain root growth and/or root hair elongation in P-deficiency-tolerant NIL6-4. That the gene expression pattern of three XTH genes distinguished groups of NILs contrasting for the presence of the *Pup1* locus could further imply a link between *Pup1* and XTH activity. However, it should be stressed that these XTHs are outside the *Pup1* region and are therefore not *Pup1* candidates. Within the *Pup1* region, only one gene with putative regulatory function was identified (OsPupK46-1; AB458444), and it remains to be tested if a link between this gene and XTH activity can be established. Furthermore, that the *Pup1* effect remains significant under well-watered conditions, even though not quite as strongly as under mild drought, indicated that the root hair phenotype would contribute to but not entirely account for the *Pup1* effect. The more general effect of XTH and other cell-wall-related genes on root growth under stress therefore warrants additional studies.

Conclusion

Many genes previously reported as inducible by P deficiency were equally induced in our study, but typically to a similar or even higher degree in Nipponbare than NIL 6-4. Considering that Nipponbare is a rather intolerant genotype when compared to a wider range of rice accessions (Wissuwa and Ae 2001a), one could argue that high-affinity P-transporter-mediated Pi uptake and efficient remobilization and redistribution of P internally are processes that do not contribute to variation in tolerance. This could be due to the crucial nature of these processes in response to the omnipresent abiotic stress P deficiency, which would consequently lead to the selection and fixation of associated genes and regulatory patterns. Allelic diversity and subsequent tolerance genes may thus be encountered for less fundamental processes such as the ability to maintain root growth or root hair elongation under a combination of drought and P deficiency. That Nipponbare is a rice variety developed for paddy rice cultivation would further support this hypothesis because drought and P deficiency do not

typically coincide in this environment and therefore were not selected for.

Materials and methods

Plant material, growth conditions, and phenotypic evaluation

Two genotypes of contrasting tolerance to phosphorus deficiency, Nipponbare and NIL6-4, were used in this study. Plants were grown in 10-l pots containing soil (highly P-fixing Andosol) from two neighboring field plots located at Tsukuba, Japan. One field plot has never received P fertilizer throughout its 40-year cropping history (−P); the other plot has been fertilized with the equivalent of 60 kg P ha^{−1} (+P). Seeds were sown directly in the soil to facilitate normal root development, and pots watered every 2–3 days to simulate “upland” growth conditions in a greenhouse. The experiment was conducted in a completely randomized design with three replications and four plants per replicate pot. Tissue samples were taken at 40 DAS; shoots (leaf blade) and roots were rapidly frozen in liquid nitrogen and stored at −70°C until analyzed. A parallel experiment was conducted for phenotypic evaluation as described above with the exception that a second sample was taken 85 DAS from plants grown in 50-l pots. P concentrations in oven-dried root and leaf samples were determined colorimetrically by the phosphovanadate method after digestion in a mixture of HNO₃, HClO₄, and H₂SO₄ (3:1:1).

For a subsequent experiment to analyze root characteristics in more detail, seeds of Nipponbare and NIL6-4 were sown in 50-l containers filled with −P soil. Pots were maintained either at field capacity (by daily addition of water) or allowed to dry by withholding water between days 23 and 42. Samples were taken 45 DAS, when plants of each genotype were near identical and were thus experiencing a similar degree of P stress. At about 55–60 DAS, genotypes already differ in P uptake and root and shoot biomass (Wissuwa 2003); therefore, the mechanism that causes the increased root biomass (which then drives further P uptake) must be present prior to this divergence (e.g., at around 45 DAS). Individual roots were washed free of soil and subsamples stored in 50% ethanol and 5% acetic acid solution until analyzed microscopically using an Olympus BX50 equipped with a DP20 digital camera (Olympus Imaging, Tokyo, Japan). Root hair length was measured on digital micrographs of first- and second-order lateral roots. Aerenchyma formation was analyzed in freehand root cross section of tissue stained with phloroglucinol/HCl and mounted in 50% glycerol.

In a similar experiment, NILs segregating for the *Pup1* locus (+*Pup1*: NILs 6-4, Y-4, 14-4; −*Pup1*: NILs 26-6, 28-10, and Nipponbare) were grown for 50 days in either P-

deficient or P-fertilized soil, and root material was sampled and immediately shock-frozen in liquid N. Some genotype information on these NILs can be found in Heuer et al. (2009), Torabi et al. (2009), and Wissuwa et al. (2002). Total RNA was extracted and used to compare the relative expression of XTH and NAD(P)H-dependent oxidoreductase genes through RT-qPCR.

Extraction of Total RNA

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instruction. In brief, frozen tissues were ground using a multibead shocker (Yasuikikai, Japan), then homogenized in the presence of a buffer containing guanidine isothiocyanate. Homogenate was applied to an RNeasy spin column and washed, and total RNA was eluted using RNase-free water. Purity of RNA was determined by OD using a spectrophotometer, and RNA integrity was determined by electrophoresis (2100 Agilent Technologies).

Microarray Slide Hybridization

The differential gene expression in each treatment was analyzed by the Rice Oligo Microarray G4138A (Agilent Technologies) which uses 60-mer oligonucleotides deduced from available cDNA clones of the rice cultivar Nipponbare (Rice Full-Length cDNA Consortium, 2003). Labeled cRNA was synthesized from 500 ng total RNA with cyanine-3-CTP (Cy3) and cyanine-5-CTP (Cy5) using a Low RNA Input Linear Amplification kit (Agilent Technologies). Hybridization solution (500 μl) containing the Cy3- and Cy5-labeled cRNA samples (1 μg each) was prepared using the in situ Hybridization Kit Plus (Agilent Technologies). Labeled cRNA samples were then hybridized to the array slide by incubating at 60°C for 17 h. Subsequently, the slide was washed twice (6× saline sodium citrate (SSC)+0.005% Triton X-102 for 10 min in room temperature and then 0.1× SSC+0.005% Triton X-102 for 5 min at 4°C) and dried with Nitrogen gas. Image file of fluorescent signal on the slide was produced with a DNA microarray scanner (Agilent Technologies). Signal intensities and their significance of the difference between the two signal intensities (*p* value of differential gene expression) were measured using the Feature Extraction software (version 8.1, Agilent Technologies).

The array experiment was undertaken using four combinations of probes (two genotypes by two P treatments) with three independent biological replicates, resulting in 12 slides for each tissue. Cy3 and Cy5 dyes were swapped in each combination. A detailed description of the microarray experimental design is shown in Supplementary Table S1, sheet “MA design.”

Data processing and statistical analysis

All analyses were based on dye-normalized signal intensities of each channel, the normalization being performed by Feature Extraction ver. 8.1 (Agilent Technologies). Significance analysis for the factor environment (–P vs +P) and genotype (Nipponbare vs NIL6-4) was performed using a mixed-model ANOVA which considers the effects of genotypes and environment conditions all at once (using a threshold $p \leq 0.001$). The model proposed by Wolfinger et al. (2001) was implemented for a loop design using R/MAANOVA (Wu et al. 2002), where the effect of genotype and environment alone was estimated by the no-interaction model:

Gene – specific fixed effect = Array + Dye + Genotype + Env.
Random effect = Array;

and genotype \times environment interactions were estimated by the model:

Gene – specific fixed effect = Array + Dye + Genotype
+ Env. + (Genotype \times Env.)
Random effect = Array

Gene annotations were based on the Rice Genome Annotation Project annotation database (TIGR5; http://rice.plantbiology.msu.edu/data_download.shtml). For probes lacking an annotation in TIGR5, several alternative annotations were considered either based on information provided by the manufacturer (Agilent) or on BLAST searches for the corresponding gene model in *Arabidopsis*. Alternatively, RAP annotation (<http://rapdb.dna.affrc.go.jp>) was used when functional annotations were not available in the previous search (TIGR and Agilent; Supplementary Table S1).

Comparison of mRNA transcript abundance using RT–PCR

Residual DNA was removed from total RNA of samples intended for quantitative PCR using the RNase-Free DNase set (Qiagen) during the RNA extraction procedure.

Around 400 ng total RNA was reverse-transcribed using Random 6 monomers and ExScript RTase of the ExScript RT reagent kit (Takara, Japan). Thermal conditions were: 42°C for 30 min and 95°C for 2 min. Subsequently, the first-strand cDNA was used as templates for PCR. PCR thermal conditions were as follows: first denaturing step at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C to 60°C for 30 s, and 72°C for 90 s, and concluded by an extension step at 72°C for 10 min.

qPCR was performed with 10 ng RT template and SYBR Premix ExTaq (Perfect Real Time, Takara, Japan), using the

Mini Opticon Real-Time PCR system (BioRad, USA). Thermal conditions were: 94°C for 10 s as the first denaturing step, followed by 94°C for 5 s, 55°C to 60°C for 10 s, and 72°C for 15 s, for 40 cycles, followed by a gradual increase in temperature from 55°C to 96°C during the dissociation stage (to monitor the specificity of each primer set). Serial dilutions of RT product were used to determine the efficiency of each primer. A rice actin (RAC1) was used as internal control. Relative expression levels between samples (the same as the microarray ones) were calculated using the delta–delta comparison and expressed as fold changes, using Nipponbare or +P soil conditions for normalization. The normalized data were analyzed by ANOVA and pairwise comparison (Tukey test) for each gene.

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