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ORIGINAL PAPER

Genetic analysis of root elongation induced by phosphorus deficiency in rice (*Oryza sativa* L.): fine QTL mapping and multivariate analysis of related traits

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Abstract Root elongation induced by phosphorus deficiency has been reported as one of the adaptive mechanisms in plants. Genetic differences were found in rice for the root elongation under phosphorus deficiency (REP), for which a distinct quantitative trait locus (QTL) was detected on the long arm of chromosome 6. Subsequently, the effect and position of the QTL, designated as *qREP-6*, were confirmed using chromosome segment substitution lines (CSSLs), in which the background of a *japonica* cultivar, 'Nipponbare' with non-REP, was partially substituted by chromosomal segments from an *indica* cultivar, 'Kasalath' with remarkable REP. Out of 54 CSSLs, two lines (CSSL28 and CSSL29) that retain a common 'Kasalath'-derived segment on the long arm of chromosome 6 showed a significantly high REP. The high REP lines also showed high adaptabilities such as enhanced tillering ability and shoot phosphorus content. Accordingly, conditional dependencies between the related traits were assessed using a graphical Gaussian model (GGM). Direct interactions between REP and root length, and between root length and tiller number were detected under P deficiency in CSSLs. Furthermore, *qREP-6* for REP and *qTNP-6* for tiller number under P deficiency were fine-mapped with an F_2 population of a cross between Nipponbare and CSSL29. A region containing *qREP-6* accounted for more than half of the phenotypic variance, the most plausible interval of which contained 37 candidate genes. The result provides a foundation for cloning of the *qREP-6* gene which will be applicable to study P deficiency-dependent response and to improve rice's adaptability to P deficiency stress.

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Introduction

Phosphorus (P), an essential element for plant growth, is often a major limiting factor for crop production because its availability in soil tends to be low due to its fixation into insoluble compounds. Consequently, phosphorus deficiency is one of the most widespread soil problems, against which plants appear to have developed several mechanisms to enhance P uptake under P deficient conditions (Raghothama and Karthikeyan 2005). Of these mechanisms, increased root growth is considered to be an effective adaptation for enhancing P uptake (Vance et al. 2003; Lópes-Bucio et al. 2003). There have been a number of reports on P deficiency-induced root elongation in plants such as *Arabidopsis* (Ma et al. 2003), barley (Steingrobe et al. 2001), horse gram (Anuradha and Narayanan 1991), and rice (He et al. 2003; Kirk and Du 1997; Wissuwa 2005). In rice, elongated roots showed higher porosity and oxygen-releasing ability (Kirk and Du 1997). Thus far, several QTLs (quantitative trait loci) analyses for P deficiency-induced root elongation have been reported for *Arabidopsis* (Reymond et al. 2006), wheat (Li et al. 2007), and rice (Shimizu et al. 2004). Although no

correlation was found between root length and a primary QTL for P uptake in P deficient upland soil (Wissuwa 2005), and no physiological evidence was found for the effect of root elongation on P deficiency tolerance (Ismail et al. 2007; Wissuwa et al. 2005), P deficiency-induced root elongation is a convenient visible marker for studying the stress-response, as reported by Svistoonoff et al. (2007).

Rice is not only one of the most important crops in the world but also a key model plant for which the entire genome has been sequenced (International Rice Genome Sequencing Project 2005). Since then, many novel tools such as genetic markers, mapped populations and genomic libraries have been developed towards comprehensive understanding and analysis of the rice genome (Sasaki 2003). These tools have enabled us to study complex traits such as stress-induced traits and to analyze the genes involved in such traits (Yano 2001). Of these novel tools, chromosome segment substitution lines (CSSLs; Ebitani et al. 2005, Ishikawa et al. 2006) are especially useful for isolating chromosomal segments of any QTLs identified in a segregating population. CSSLs are developed by repeated backcrossing of a background cultivar into a substituted cultivar, so that each of the resulting lines contains chromosomal segments that are isolated from a substituted variety and incorporated into a background variety. Thus, a set of CSSLs may cumulatively cover the entire genome of a substituted cultivar, with partially overlapping segments. For rice, several sets of CSSLs have been developed and released by the Rice Genome Resource Center in Japan (RGRC 2004). By using these new tools, a genome-wide screening of useful genes is now feasible to produce cultivars that are tolerant to problem soils, as proposed by Ismail et al. (2007).

In our previous study, a distinct QTL for root elongation under P deficiency (REP) was detected on the long arm of chromosome 6 using recombinant inbred lines (RILs) derived from *Oryza sativa* cv. 'Gimbozu' and 'Kasalath' (Shimizu et al. 2004); in the present study, it is designated as *qREP-6*. The REP is a relative trait obtained by the ratio of root length under P deficient condition to root length under the control condition. This relative trait has a high level of genetic variance, because root lengths under the two kinds of conditions may be controlled by mutually independent loci. Because REP is a trait induced by P deficiency stress, cloning of *qREP-6* would provide a clue to its responsive pathway. Thus, we must first perform a genetic analysis of REP under a low level of genetic variance.

The objective of this study is to confirm the effect of 'Kasalath' alleles within the *qREP-6* region by utilizing a set of CSSLs. CSSLs are particularly useful for genetic analyses of such relative traits as REP because their phenotypic variances are reduced under homogeneous genetic background. We also analyzed other stress-related traits, i.e., tiller number and shoot P content under P deficiency. Because some CSSLs with a high REP value showed a significant increase in tiller number and shoot P content, partial correlation coefficients between REP and other traits were studied to find conditional dependencies among the traits. We applied a graphical Gaussian model (GGM) to distinguish between direct and indirect interactions among the traits. Finally, fine QTL mapping was conducted using an F_2 population derived from a cross between 'Nipponbare' and a CSSL containing *qREP-6* alleles of 'Kasalath'. Candidate regions for the REP and tiller number under P deficiency were surveyed to pinpoint responsible genes. The results provided an essential step for cloning of the gene to study P deficiency-dependent response and to enhance the tolerance of rice to phosphorus deficiency.

Materials and methods

Plant materials

First, chromosome segment substitution lines (CSSLs) provided by the Rice Genome Resource Center (RGRC 2004) were screened for root elongation under P deficiency (REP) and other related traits. Their parental lines of *Oryza sativa* cv. 'Kasalath' and 'Nipponbare' exhibited elongation and non-elongation of roots, respectively, under P deficiency (Shimizu et al. 2004). The CSSLs, each of which contains 'Kasalath'-derived chromosomal segments, have been developed by repeated backcrossing of 'Nipponbare', which provided the genetic background of CSSLs. A set of 54 CSSLs, each of which contains one major segment, was chosen to cover the entire genome of 'Kasalath' with partial overlapping. The mean length of substituted segments was 26.2 cM and the mean number of segments per line was 3.1, given that each CSSL contained several minor segments in addition to a major segment. A total of 214 RFLP markers covering the saturated linkage map of 1,503 cM were recorded for the CSSLs with their map positions and are available from RGRC (2004).

Second, one line of CSSLs, namely CSSL29, which showed the highest REP, was crossed with the background parent of 'Nipponbare' and a total of 752 F_2 seedlings obtained from the cross were screened for a fine mapping of REP and the number of tillers under P deficiency. In the test of the F_2 population, root length under P deficiency was used to estimate REP, which is defined as the ratio of root eongation under P deficient solution to that under the control condition, because each genotype in an F_2 population cannot be grown under both P deficient and control conditions.

Methods of growing plants under nutritional solution

Rice seedlings were screened with Yoshida's nutritional solution (Yoshida et al. 1976) as earlier reported (Shimizu

et al. 2005). The nutritional solution was composed of following constituents: 1.43 mM NH_4NO_3, 0.016 (P deficient) or 0.32 (control) mM NaH_2PO_4, 0.51 mM K_2SO_4, 1.00 mM $CaCl_2$, 1.64 mM $MgSO_4$, 9.11 μM $MnCl_2$, 0.07 μM $(NH_4)_6Mo_7O_{24}$, 18.49 μM H_3BO_3, 0.15 μM $ZnSO_4$, 0.16 μM $CuSO_4$, and 35.77 μM $FeCl_3$. Surface-sterilized seeds were soaked in sterilized distilled water at 20°C for 48 h and then incubated at 30°C for 48 h. The germinated seeds were placed on a polystyrene plate of 61.0 × 40.8 cm with 280 holes, which was floated in a plastic container containing 80 L nutritional solution. The nutritional solutions were changed weekly and the pH of the solution was adjusted daily to 5.0 with 1 N NaOH or HCl. The CSSLs were screened under both P deficient and control conditions. The number of tillers and root length were recorded after five weeks. REP was then given by a ratio of the root length in P deficient condition to that in the control. In contrast, the F_2 plants were screened only under the P deficient condition, so root length under P deficiency was measured as REP.

The screening was conducted in a daylight greenhouse at the Experimental Farm of Nihon University, Fujisawa, Japan. The mean temperatures during the experiment were 28.1 for CSSLs and 27.8°C for F_2 seedlings.

Screening, measurements and analysis of CSSLs

Two hundred and eighty plants consisting of five plants for each of the 56 lines per plot (two parental lines and 54 CSSLs) were screened in an 80-L container. Because two phosphorus levels each with two replicates, i.e., 2 × 2 = 4 plots, were set up as experimental plots, a total of 20 plants per line were screened. After the screening, shoot samples were harvested, dried at 80°C for 48 h, and weighed. Five plant samples per line per treatment were divided into two parts for replicated measurements. Ground samples were digested with mixed acids and filtered with Whatman filter No. 42 (Whatman International, Maidstone, UK). The phosphorus concentration (μmol g^{-1} DW) in each shoot sample was determined colorimetrically by a molybdate-vanadate method according to Shimizu et al. (2005). Shoot P content (μmol per plant) was calculated as the product of shoot P concentration and dry weight of the shoot.

For the statistical analysis of REP, tiller number, and shoot P content, Dunnett's test was used to detect significant differences between each CSSL and the background variety, 'Nipponbare'. A function of 'glht' with the Dunnett option in a 'multcomp' library was performed using the statistical package R ver. 2.6.1. (R Development Core Team 2007). Because a P value of Dunnett's test was adjusted for a family wise error rate, $P < 0.05$ was set as the significance level. The normality of distribution of traits was assessed using the D'Agostino-Pearson K^2 test, in which the skewness and kurtosis of the distribution were used for calculations (Zar 1999).

Graphical Gaussian model for conditional relationships among traits

Associations between REP and other traits in CSSLs were analyzed to assess the contribution of REP to the tolerance to P deficiency stress. In general, an association between two biological characters is estimated with a simple correlation coefficient, i.e., a Pearson's r (Zar 1999), and their pairwise associations are indicated by a correlation matrix. However, this may lead to some spurious judgements because the simple correlation can detect marginal associations but cannot distinguish direct interactions from indirect ones. In particular, a marginal association is spurious in the case of an indirect effect in which, for example, trait *A* and trait *B* correlate only via trait *C*.

To avoid such a misunderstanding, we used a graphical Gaussian model (GGM) (Edwards 2000). In a GGM, partial correlation coefficients are used to distinguish conditional associations from marginal ones with significant conditional dependencies visualized by an undirected graph, on which variables are indicated by the vertices and significant conditional dependencies between variables are encoded by edges. In a GGM, an absence of an edge between vertices is defined by setting the partial correlation coefficients at zero (non-significant). Graphical Gaussian models have been applied in various fields, as reviewed by Jordan (2004), and are useful for analyzing interactions among multivariate data such as multiple related traits. Significant conditional associations between REP and other five traits such as absolute root length, tiller number, dry weight of the shoot, shoot P concentration (μmol g^{-1} DW), and shoot P content (μmol per plant) were assessed by a GGM using a software program, MIM ver 3.2.0.6 (Edwards 2007) with an information criteria of Bayesian Information Criteria (BIC) from (Schwarz 1978). Because there were six traits, the full graph could have 15 edges resulting from 6 × 5/2, and there were possible models with the presence or absence of 15 edges, amounting in total to 2^{15} = 32,768 models. Of all the models, the least BIC model was selected as the best-fitting GGM having parsimonious edges. According to Edwards (2000), the GGM with the least BIC will tend to be simpler than the GGM with other information criteria such as Akaike Information Criteria (AIC, Akaike 1974). GGM analyses were conducted in both P deficient and control conditions.

Fine mapping of root length and tiller number under P deficiency

After the five-week screening of F_2 seedlings under P deficient solution, fresh leaf blades of 3–4 cm^2 were sampled

from each plant to extract genomic DNA according to Collard et al. (2007) with slight modification as follows. A piece of leaf placed into a 1.5 mL tube was mixed with a zirconia bead and 0.6 mL of extraction buffer consisting of 50 mM Tris-HCl, 25 mM EDTA, 300 mM NaCl, and 1% SDS at pH 8.0. The supernatant was extracted with chloroform and isoamyl alcohol (24:1) and precipitated with 2-propanol. The pellet was washed with 70% ethanol, air-dried, and resuspended in a dilution buffer of 1 mM Tris-HCl and 0.1 mM EDTA buffer at pH 8.0.

Polymerase chain reaction (PCR) was carried out in a reaction volume of 7.5 μL containing 50-100 ng template DNA, 0.2 μM of each primer, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 1× PCR buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris, pH 8.8, 0.01% Tween-20], and 0.375 unit of BIO Taq polymerase (BIOLINE, London, UK). The PCR profile was as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and 7 min at 72°C for the final extension. The resulting PCR products were run on 8% polyacrylamide denaturing gels using the HEGS system as described by Shimizu et al. (2005). The simple sequence repeats (SSR) markers showing polymorphism between the parents were used to analyze the F_2 population. A linkage map was constructed using MAPMAKER (Lander et al. 1987) based on Haldane's map function. QTL analyses by composite interval mapping were applied using Windows QTL Cartographer 2.5 (Wang et al. 2007). A threshold LOD score was calculated by permutation of 1,000 replicates at a significance level of 0.05.

Databases for rice genome, gene annotation and QTLs

The rice genome sequence released by the IRGSP build 4.0 (IRGSP 2007) was used to estimate the physical distance of a substituted segment and the positions of DNA markers. To search predicted genes in the genomic region, RAP2 released by the Rice Annotation Project (2008) was used. The Gramine QTL database (2008) was used for surveying previously reported QTLs.

Results

Confirmation of chromosomal segments responsible for REP

To identify a chromosomal segment containing Kasalath's allele for REP, a set of CSSLs was screened under P deficient and control conditions in duplicate. Mean shoot P concentration of CSSLs under P deficiency was about one-eighth of that of the control (17.4 ± 1.9 vs. 129.8 ± 13.2 μmol g^{-1} DW) (see Electronic Supplementary Material) and all P deficient plants showed lower concentrations than the critical limit of 32 μmol g^{-1} DW (1.0 mg g^{-1} DW; Yoshida et al. 1976). The D'Agostino-Pearson K^2 test showed that the distribution of REP among CSSLs followed a normal distribution with a mean of 1.23 ± 0.12 (SD) at $P = 0.39$ (Fig. 1). The REP of CSSL28 and CSSL29, which exceeded 1.55, had a one-tailed probability of 0.005 in the normal distribution of $N(1.23, 0.12^2)$. Dunnett's test also revealed significance at an adjusted P value of <0.024 (Table 1). They had a common substituted segment on chromosome 6 and the genetic length between the common markers of R2549 and R2071 was 21.8 cM, corresponding to 3.7 Mbp of the rice genome sequence (Fig. 2).

Relationship of REP with tiller number and shoot P content

The relationship between REP and tiller number was examined because reduction in tiller number is a typical symptom of P deficiency stress. All of the CSSLs had mean tiller numbers of more than 1.0 under the control condition (Fig. 3c), while 69% of the CSSLs had a single tiller under P deficiency (Fig. 3a). There was a significant positive correlation ($r = 0.538$) between REP and tiller number under P deficiency. Only three lines, i.e., CSSL28, CSSL29, and CSSL39, with the highest REP had mean tiller numbers exceeding two. There were six lines showing a significant increase in tiller number (Table 1).

Shoot P content was surveyed because it is also a P deficiency stress-related trait. The correlation between REP and shoot P content was positively significant at the 1% level under P deficiency (Fig. 3b), but not significant under the control condition (Fig. 3d). Dunnett's test revealed five lines showing significantly high shoot P content under P deficiency (Table 1). CSSL29 and CSSL39 were the highest lines and CSSL28 showed non-significant but the sixth highest shoot P content. For further analysis of REP and P

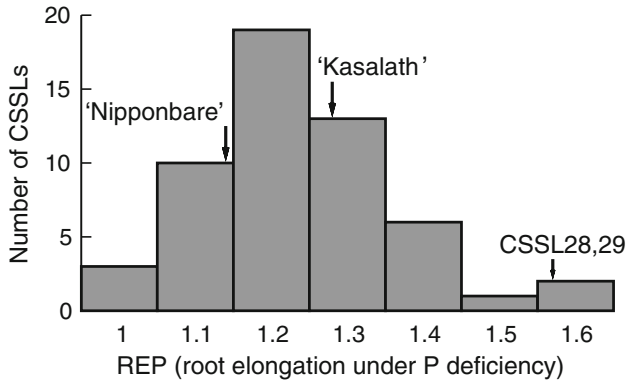

Fig. 1 Histograms of CSSLs for root elongation under P deficiency (*REP*). REP is indicated by relative root length (P deficiency/control). CSSL28 and CSSL29 are significantly separated by Dunnett's test (See Table 1) and *Z* test (Zar 1999) from other CSSLs

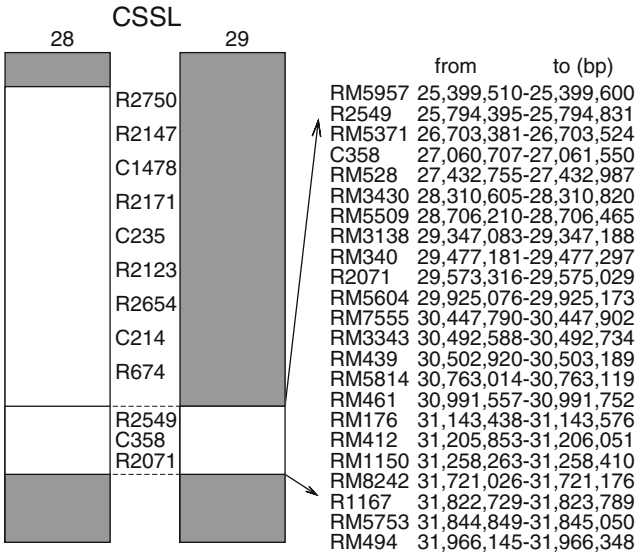

Fig. 2 Diagrammatic representation of CSSL28 and CSSL29 genotypes on chromosome 6. Chromosomes are represented by *closed rectangles* and substituted segments derived from 'Kasalath' are indicated by *open rectangles*. RFLP markers within substituted segments are listed in an intermediate position between the chromosomes. SSR markers used in the fine mapping (see also Fig. 5) are indicated with respective positions in the rice genome sequence of IRGSP build 4.0 on the right side

nutrition under P deficiency, shoot P content was divided by P concentration and dry weight, and their conditional associations were analyzed by using a GGM.

Graphical Gaussian model of REP and related traits

Conditional associations between REP and other five traits such as tiller number (TN), shoot P content (Pct), absolute root length (RL), dry weight of the shoot (DW) and shoot P concentration (P) were assessed by a GGM in CSSLs under both P deficient and control conditions. These results were compared with those of correlation matrix (COR) which has been generally used for representing multivariative relationships.

The GGM under P deficiency detected significant associations between traits as seven edges, with each edge indicating a significant association and a missing edge indicating a conditional independence between traits (Fig. 4a). The number of edges in GGM was four fewer than those in COR (Fig. 4b). Significance of five edges representing REP and TN, REP and DW, REP and Pct, RL and Pct, DW and TN in COR were rejected by GGM. To the contrary, GGM detected a new edge between P and Pct which was not detected in COR and absolute values of partial correlation coefficients among P, Pct and DW were very strong i.e., more than 0.97 (Fig. 4a). The result indicated that GGM was so informative to survey a computational relationship because P content (Pct) was calculated as "dry weight of the shoot (DW) × P concentration (P)".

In case of control condition, GGM and COR detected five significant edges (Fig. 4c, d). The association between P and Pct was detected only in GGM and the superiority of GGM was also supported. Strong absolute values of partial correlation coefficients among P, Pct and DW, i.e., more than 0.86 were also observed. There was no significant association of REP with other traits under contorl condition (Fig. 4c, d). The observed associations between REP and RL, between RL and TN, and between RL and DW were P deficiency-dependent (Fig. 4a, d) and that corresponded to the P deficiency-response of REP. The association between RL and TN under P deficiency can be genetically confirmed by further fine mapping.

Table 1 List of each mean value ± standard deviation (SD) of the top six CSSLs for REP, tiller number, and shoot P content under P deficiency

REP[a] (Pdef/Con)			P deficiency					
			Tiller number			Shoot P content[b] (μmol per plant)		
	Mean ± SD	P^c		Mean ± SD	P^c		Mean ± SD	P^c
Nipp[d]	1.14 ± 0.003	–	Nipp[d]	1.00 ± 0.00	–	Nipp[d]	9.53 ± 2.73	–
CSSL**29**	1.55 ± 0.243	0.023*	CSSL**29**	3.00 ± 0.67	0.000**	CSSL**29**	16.03 ± 0.16	0.000**
28	1.55 ± 0.119	0.024*	**39**	2.43 ± 0.98	0.000**	**39**	14.17 ± 4.20	0.013*
39	1.47 ± 0.101	0.129	**28**	2.14 ± 0.69	0.000**	3	13.73 ± 0.55	0.037*
38	1.43 ± 0.126	0.275	3	1.80 ± 0.92	0.000**	32	13.66 ± 2.71	0.042*
4	1.39 ± 0.111	0.489	40	1.70 ± 0.67	0.000**	40	13.65 ± 1.40	0.043*
32	1.37 ± 0.081	0.527	44	1.67 ± 0.87	0.001**	**28**	13.12 ± 3.17	0.125

[a] REP is a ratio of the root length in P deficient condition to that of the control

[b] Shoot P content (μmol per plant) is caluculated as shoot P concentration × dry weight of the shoot

[c] *p* is a probability value with Dunnett's test which is adjusted to control for the family wise error rate with multiple comparisons. Significant differences from 'Nipponbare' at the 5% and 1% levels are indicated by * and **, respectively

[d] Nipp is the abbreviation of 'Nipponbare', the background variety used as a control

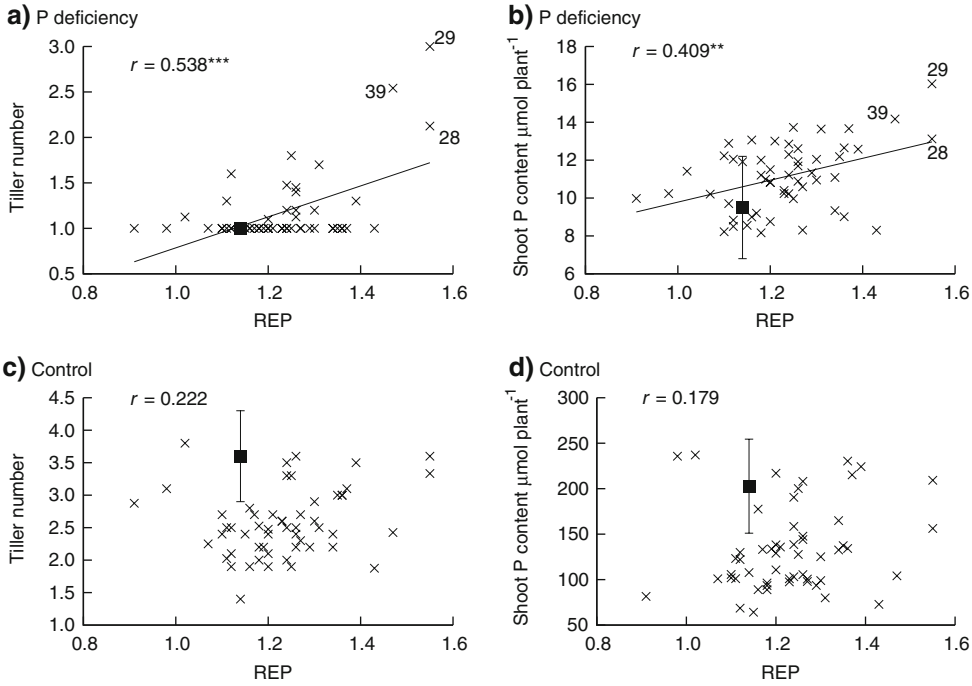

Fig. 3 Correlations (*r*) of REP with tiller number (**a**, **c**), and shoot P content (**b**, **d**) under P deficiency (**a**, **b**) and control (**c**, **d**) conditions in CSSLs. Shoot P content was calculated as 'shoot P concentration' × 'dry weight of the shoot'. Plots of CSSL28, CSSL29, and CSSL39 under P deficiency are indicated. ** and *** indicate significant correlation at the 1 and 0.1% levels, respectively. A *square* and *vertical bar* in each figure indicates the mean value ± SD for 'Nipponbare' as indicated in Table 1. The mean value ± SD for 'Kasalath' is as follows: 1.28 ± 0.004 for REP, 1.3 ± 0.5 (*Pdef*), and 4.2 ± 1.3 (*Con*) for tiller number and 14.8 ± 4.5 (*Pdef*) and 426.9 ± 29.6 (*Con*) for shoot P content

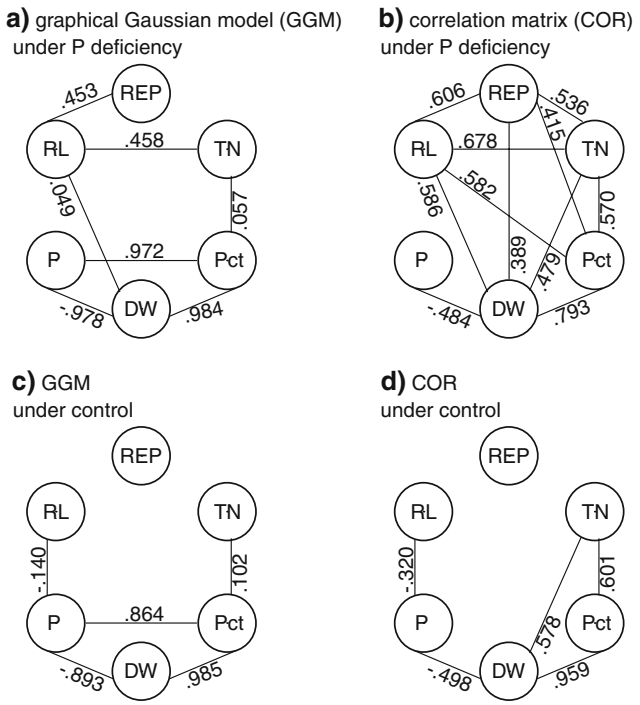

Fig. 4 Undirected graphs representing associations of REP and other related traits in CSSLs by graphical Gaussian model (*GGM*) (**a**, **c**) and correlation matrix (*COR*) (**b**, **d**) under P deficiency (**a**, **b**) and control (**c**, **d**) in CSSLs. In the graphs, traits are indicated by the vertices (open circles), while significant correlations or partial correlations between traits are indicated by edges with their coefficients. "*RL*", "*TN*", and "*DW*" indicate absolute root length, tiller number, and dry weight of the shoot, respectively and "*P*" and "*Pct*" indicate P concentration and P content in the shoot, respectively

Fine QTL mapping for root length and tiller number under P deficiency

A linkage map was constructed using 752 plants of the F_2 population from a cross of 'Nipponbare' and CSSL29, which showed the highest REP value and tiller number under P deficiency. There were 19 polymorphic SSR markers on a substituted segment of chromosome 6 and its map length was 23.3 cM with a mean interval of 1.29 ± 1.24 (SD) cM in Haldane's map function (Fig. 5). The mapped segment corresponds to a sequence position from 25.39 to 31.97 Mbp of the genome sequence of IRGSP build 4.0 (2007).

Fine mapping for *qREP-6* was conducted with composite interval mapping. The computed threshold was an LOD of 2.1 and almost all mapped segments had LOD scores greater than the threshold (Fig. 5). The highest LOD score between RM439 and RM5814 was 95.2 and it accounted for 54.5% of phenotypic variance for which an additive effect of 3.4 (cm) was estimated with no dominance. Although a hypothesis of normality of raw data was rejected for REP at the 1% level ($P = 9.1 \times 10^{-8}$) and a distribution of log-transformed data followed normality at $P = 0.38$, raw data were used for the analysis because both QTL profiles between non- and log-transformed data were correlated at R^2 of 99.98% and there was no positional difference in LOD peak.

QTL mapping was also conducted for tiller number (TN). Of the F_2 plants, 705 had a TN of 1, 35 had a TN of 2,

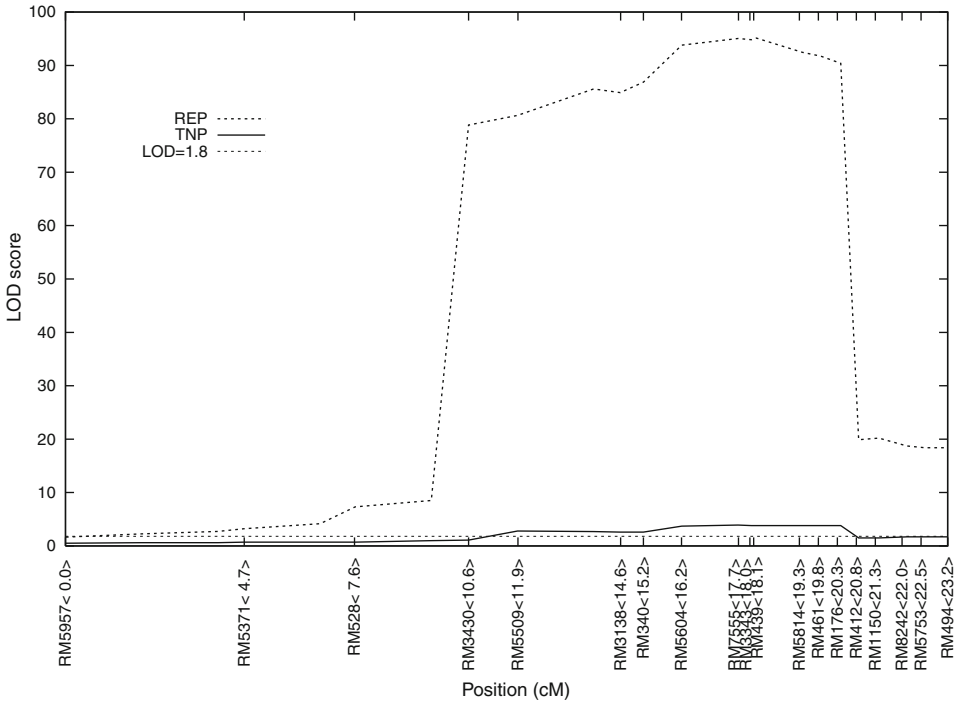

Fig. 5 QTL profiles for REP and TNP (tiller number under P deficiency) using 752 plants of the F_2 population of Nipponbare/CSSL29 hybrid. The *horizontal axis* indicates LOD scores and the *vertical axis* indicates genetic positions within the substituted segment on the long arm of chromosome 6 by Halden centimorgan (cM). Mapped SSR markers and their genetic distance from the upper side of the segments (cM) are listed at the left side and their positions are indicated by *horizontal bars*. The *vertical dotted* line of LOD = 1.8 is the threshold of QTL for TNP. Genome positions of SSR markers are listed in Fig. 2

and 12 had a TN of 3, and the distribution was strongly skewed to 1. Because no effective transformation was found among log-, reciprocal-, square- and square root- transformations, raw data were used for the analysis. The region from RM5509 to RM176 had LOD scores greater than the threshold of 1.8 and its length was 8.4 cM. The highest LOD peak between RM7555 and RM3343 was 3.9, which accounts for only 3.6% of the phenotypic variance (Fig. 5). LOD profiles between QTLs for both REP and tiller number were correlated at R^2 of 83.1%.

The pleiotropic effect of the genetic locus controlling for tiller number on that for root length under P deficiency was assessed by one-way (analysis of variance). F_2 plants with TN = 1 had a mean ± SD root length of 16.2 ± 3.1 (cm); those with TN = 2 had a root length of 18.2 ± 3.4 (cm); and those with TN = 3 had a root length of 23.3 ± 3.1 (cm). There was significant difference in root length among tiller numbers at $P = 1.1 \times 10^{-15}$ (Fig. 6). Tiller number accounted for 8.5% of the phenotypic variance of REP and only one-fourth of that by RM439 (43.9%), which was the nearest marker of *qREP-6*.

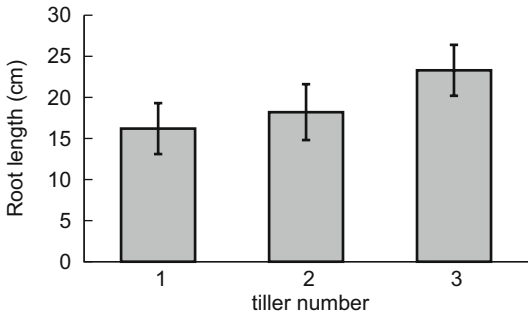

Fig. 6 Root length classified by tiller number in the F_2 population of Nipponbare/CSSL29 hybrid. The *horizontal axis* indicates classification by tiller number from 1 to 3 and the *vertical axis* indicates mean root length (cm) of classified F_2 plants. Standard deviation of root length is represented by a *vertical bar*

Discussion

The QTL for REP (Root Elongation under Phosphorus deficiency), i.e., *qREP-6*, is considered to be a visible sign of a plant's positive adaptation to P deficiency and a key to improving the tolerance of plants to P deficiency. This QTL was earlier detected by using recombinant inbred lines of 'Gimbozu' and 'Kasalath' (Shimizu et al. 2004) and confirmed in the present study by screening of chromosome segment substitution lines (CSSLs). Out of 54 CSSL lines, CSSL28 and CSSL29 showed significantly higher REP than the background parent of 'Nipponbare'. These lines were confirmed to have a common substituted segment on the long arm of chromosome 6 in accordance with the preceding QTL analysis. Thus, the screening of CSSLs proved to be instrumental for detecting a chromosomal segment that is responsible for an intricate phenotype.

From statistical analyses, Dunnett's test was shown to be effective for detecting differences between substitution lines and a background parent, because it can control the family wise error rate easily, even in the multiple comparisons. The level of significance in the detection of CSSL29 was also confirmed by a GGM or fine mapping.

Simple correlations of REP with both tiller number and shoot P content were significantly positive in CSSLs under P deficient condition. GGM analysis, however, revealed that these correlations were marginal associations. Instead, three direct associations between REP and absolurte root length (RL), between RL and tiller number (TN), and between TN and shoot P content (Pct) were detected. Of these, two associations between REP and RL and between RL and TN were P deficiency specific and that suggested the stress-response of REP and its direct effect on root length and indirect effect on other traits via root length.

The association between REP and tiller number via root length was simultaneously confirmed by the lines of CSSL28, CSSL29 and CSSL39, which were significantly separated by Dunnett's test. These lines had the highest rates of root elongation, highest number of tillers, and higher levels of P content in the shoot compared to the other lines. The correlative induction of these kinds of traits has also been reported by other researchers. For example, a near isogenic line of 'Nipponbare' containing a 'Kasalath' segment on chromosome 12, where a P deficiency tolerance QTL of *Pup1* was mapped, showed high P content, high tillering ability, and high root growth under P deficient upland condition (Wissuwa and Ae 2001; Wissuwa et al. 2002). *Pup1* is not yet cloned and its precise mechanism is still unknown, but Wissuwa (2005) suggested that an observed increase of root growth was a subsequent factor and that external P uptake efficiency was the primary cause of *Pup1*. In the present study, REP may also be a subsequent trait after efficient P uptake and so the high P content lines concomitantly showed highest tiller numbers. Yi et al. (2005) reported that a transgenic rice strain containing *OsPTF1* of a P deficiency-induced transcription factor, showed approximately 30% higher tillering ability, higher P content, and higher root biomass under P deficient nutritional solution. *OsPTF1* positioned on the short arm of chromosome 6 (on the opposite side of REP), seemed to confer the tolerance by directly regulating responsible genes, because its overexpression altered the expression of 158 genes whose promoter regions contained putative recognition motifs of *OsPTF1* and which included P metabolism-related genes such as transporter and phosphatase. Becasue REP was observed in nutritional solution by limiting P below the threshold (Shimizu et al. 2004), it may also be indicative of a trigger of genes under stress. There have been reports on P deficiency-response genes in rice (Wasaki et al. 2003, 2006), but their roles in the P-pathways are still unclear. Positional cloning of genes controlling REP may reveal a novel stress-response pathway. Even if REP is a subsequent trait of another tolerant mechanism, root elongation under P deficiency is a visible trait and convenient for positional cloning. Because REP was observed in anaerobic conditions contray to *Pup1* (Wissuwa and Ae 2001), then cloning of REP may contribute to P deficiency tolerance of rice in lowland fields.

In the present study, the earlier identified region for *qREP-6* was confirmed within the substituted segment on the long arm of chromosome 6 by fine QTL mapping with 752 plants from an F_2 population that was derived from a cross of CSSL29 and the background cultivar 'Nipponbare'. The LOD peak for the root length under P deficiency was mapped on the sequence position from 30.50 to 30.76 Mbp on chromosome 6 and it corresponded to the previously mapped QTL (Shimizu et al. 2004). The peak accounted for more than half of the phenotypic variance, where the Kasalath allele conferred a 3.4 cm longer root per allele in an additive way.

It is interesting that there was still a broad region having an LOD score of more than 78.8, which equaled 82% of the highest peak. Several possibilities may explain this: (1) A typical quantitative trait such as root length showed a continuous segregation in an F_2 population and information on recombinants between marker genotype and phenotype was not available. Fine QTL mapping was then conducted using composite interval mapping and as a result, the mapping accuracy was lower than with fine mapping using precise recombinant information. (2) The analyzed trait in F_2, i.e., root length under P deficiency, was complicated, being jointly affected by root length under control condition and root elongation induced by P deficiency. As indicated by the fact that the donor parent of CSSL29 showed a significantly longer root under control condition than the recipient parent of Nipponbare (see Electronic Supplementary Material), the composite nature would increase the phenotypic variance and as a result, the mapping accuracy would decrease. (3) There was a possibility of gene cluster. The region was flanked by markers RM3430 and RM176 and contained 297 RAP2 loci from Os06g0664300 to Os06g0713800 (RAP-DB 2008). Such composition of the trait would increase the number of involved genes. (4) Severe recombination suppression was not observed. The mean recombination frequency estimated from Fig. 2 and Fig. 5 was 271.5 ± 140.3 (SD) kb/cM in the substituted segment, approaching the 277 kb/cM that was calculated as the genome-wide average frequency in rice (Dinka et al. 2007). According to the Gramine QTL database (2008), there was no QTL for root length onto the substitution segment, but a QTL for root penetration (Ray et al. 1996) was detected. A scientific note by Wissuwa (2006) described a QTL for root length in P starvation corresponding to *qREP-6* using two kinds of recombinant inbred lines, Nipponbare/Kasalath and Koshihikari/Kasalath. Because of the P stress-response of root length reported by Shimizu et al. (2004) and confirmed by our CSSL-screening, further progeny testing using candidate recombinants is required for the precise mapping of *qREP-6*.

The genomic region for tiller number in P deficiency was also analyzed in the fine mapping. It is interesting that the LOD peaks for tiller number and REP were located on slightly different genomic regions; accordingly, these traits were likely to be closely linked to each other but to be controlled by different genes. This assumption was also supported by the fact that the number of tillers under P deficiency and the LOD peak for *qREP-6* accounted for only 8.5 and 54.5% of the phenotypic variance of REP, respectively. The marker interval showing the highest LOD peak for tiller number was mapped on the genomic sequence from 30.45 to 30.49 Mbp of chromosome 6 but it accounted for only 3.6% of its phenotypic variance. This may be due to the skewed distribution of tiller number in which 94% of F_2 plants had only one tiller under P deficiency. In the Gramine QTL database (2008), there were two reports in which the QTLs for tiller number were detected on the corresponding region (Kobayashi et al. 2003; Yan et al. 1998). However, precise comparisons of these results with ours were not possible, because those QTLs were not obtained by fine mapping and a candidate gene was not yet isolated. Therefore we tentatively designated this QTL as *qTNP-6* (tiller number under P deficiency). To determine whether *qTNP-6* and the effect of *qREP-6* are related, further fine mapping of *qREP-6* should be performed before mapping of *qTNP-6*, because the distribution of tiller number was skewed under P deficiency, particularly with young seedlings.

Further analyses are necessary to isolate *qREP-6* because the most plausible interval between markers RM439 and RM5814 still contains 37 of the RAP2 loci or assumed genes. In a previous report (Shimizu et al. 2004), we focused on REP as a stress-response trait. There is a possibility that a candidate for *qREP-6* is one of genes involved in P deficiency-response pathway because a transcription factor that was up-regulated by P deficiency was detected in the candidate region by microarray analysis of rice (Shimizu et al., unpublished). If root elongation is a subsequent phenotype caused by stress-response, an observed adaptability of CSSL29 could be explained by stimulation of the stress-response pathway. Further positional cloning of *qREP-6* will reveal whether *qREP-6* is a regulatory factor or one of the regulated genes. For the next step, additional mapping of F_2 plants has been processed and testing of their progeny will be conducted to find appropriate recombinants that may pinpoint an exact locus. A cloned *qREP-6* would provide a key tool for studying P deficiency stress-response and for improving the adaptability of rice to P deficiency stress through marker-assisted selection or genetic modification.

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References

Akaike H (1974) A new look at the statistical model identification. IEEE Trans Automat Contr 19:716–723

Anuradha M, Narayanan A (1991) Promotion of root elongation by phosphorus deficiency. Plant Soil 136:273–275

Collard BCY, Das A, Virk PS, Mackill DJ (2007) Evaluation of 'quick and dirty' DNA extraction methods for marker-assisted selection in rice (*Oryza sativa* L.). Plant Breed 126:47–50

Dinka SJ, Campbell MA, Demers T, Raizada MN (2007) Predicting the size of the progeny mapping population required to positionally clone a gene. Genetics 176:2035–2054

Ebitani T, Takeuchi Y, Nonoue Y, Yamamoto T, Takeuchi K, Yano M (2005) Construction and evaluation of chromosome segment substitution lines carrying overlapping chromosome segments of indica rice cultivar 'Kasalath' in a genetic background of japonica elite cultivar 'Koshihikari'. Breed Sci 55:65–73

Edwards D (2000) Introduction to Graphical Modelling, 2nd edn. Springer-Verlag, New York

Edwards D (2007) The MIM Web Site. http://www.hypergraph.dk. Cite 6 Aug 2007

Gramine QTL Database (2008) Cold Spring Harbor Laboratory and Cornell University, USA. http://www.gramine.org. Cited 4 Jan 2008

He Y, Lian H, Yan X (2003) Localized supply of phosphorus induces root morphological and architectural changes of rice in split and stratified soil cultures. Plant Soil 248:247–256

Project International Rice Genome Sequencing (2005) The map-based sequence of the rice genome. Nature 436:793–800

IRGSP (2007) International Rice Genome Sequencing Project, Japan. http://rgp.dna.affrc.go.jp/E/IRGSP/Build4/build4.html. Cited 13 Dec 2007

Ishikawa S, Ae N, Yano M (2006) Chromosomal regions with quantitative trait loci controlling cadmium concentration in brown rice (*Oryza sativa*). New Phytol 168:345–530

Ismail AM, Heuer S, Thomson MJ, Wissuwa M (2007) Genetic and genomic approaches to develop rice germplasm for problem soils. Plant Mol Biol 65:547–570

Jordan M (2004) Graphical Models. Stat Sci 19:140–155

Kirk GJD, Du LV (1997) Changes in rice root architecture, porosity, and oxygen and proton release under phosphorus deficiency. New Phytol 135:191–200

Kobayashi S, Fukuta Y, Sato T, Osaki M, Khush GS (2003) Molecular marker dissection of rice (*Oryza sativa* L.) plant architecture under temperate and tropical climates. Theor Appl Genet 107:1350–1356

Lander ES, Green P, Abrahamson J, Barow A, Daly MJ, Lincoln SE, Newbug L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Li Z, Ni Z, Peng H, Liu Z, Nie X, Xu S, Liu G, Sun Q (2007) Molecular mapping of QTLs for root response to phosphorus deficiency at seedling stage in wheat (*Triticum aestivum* L.). Prog Nat Sci 17:1177–1184

Lópes-Bucio J, Cruz-Ramírez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 6:280–287

Ma Z, Baskin TI, Brown KM, Lynch JP (2003) Regulation of root elongation under phosphorus stress involves changes in ethylene responsiveness. Plant Physiol 131:1381–1390

Raghothama KG, Karthikeyan AS (2005) Phosphate acquisition. Plant Soil 274:37–49

RAP-DB (2008) Rice Annotation Project Database, Japan. http://rapdb.dna.affrc.go.jp. Cited 9 Jan 2008

Ray JD, Yu L, McCouch SR, Champoux MC, Wang G, Nguyen HT (1996) Mapping quantitative trait loci associated with root penetration ability in rice (*Oryza sativa* L.). Theor Appl Genet 92:627–636

R Development Core Team (2007) R: A Language and Environment for Statistical Computing. http://www.r-project.org. Cited 26 Nov 2007

Reymond M, Svistoonoff S, Loudet O, Nussaume L, Desnos T (2006) Identification of QTL controlling root growth response to phosphate starvation in *Arabidopsis thaliana*. Plant Cell Environ 29:115–125

RGRC (2004) Rice Genome Resource Center, Japan. http://www.rgrc.dna.affrc.go.jp/stock.html

Rice Annotation Project (2008) The rice-annotation project database (RAP-DB): 2008 update-. Nucleic Acid Res 36:D1028–D1033

Sasaki T (2003) Rice genome analysis: understanding the genetics secrets of the rice plant. Breed Sci 53:281–289

Schwarz G (1978) Estimating the dimension of a model. Ann Statist 6:461–464

Shimizu A, Guerta CQ, Gregorio GB, Kawasaki S, Ikehashi H (2005) QTLs for nutritional contents of rice seedlings (*Oryza sativa* L.) in solution cultures and its implication to tolerance to iron-toxicity. Plant Soil 275:55–64

Shimizu A, Yanagihara S, Kawasaki S, Ikehashi H (2004) Phosphorus deficiency-induced root elongation and its QTL in rice (*Oryza sativa* L.). Theor Appl Genet 109:1361–1368

Steingrobe B, Schmid H, Claassen N (2001) Root production and root mortality of winter barley and its implication with regard to phosphate acquisition. Plant Soil 237:239–248

Svistoonoff S, Creff A, Reymond M, Sigoillot-Claude C, Ricaud L, Blanchet A, Nussaume L, Desnos T (2007) Root tip contact with low-phosphate media reprograms plant root architecture. Nature Genet 39:792–796

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

Wang S, Basten CJ, Zeng Z-B (2007) Windows QTL Cartographer 2.5. Department of Statistics, North Carolina State University, Raleigh, NC. http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

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

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

Wissuwa M (2005) Combining a modelling with a genetic apporach in establishing associations between genetic and physiological effects in relation to phosphorus uptake. Plant Soil 269:57–68

Wissuwa M (2006) Comparative QTL mapping of root length in the Nipponbare/Kasalath and Koshihikari/Kasalath mapping populations. Int Rice Res Notes 31:53–54

Wissuwa M, Ae N (2001) Further characterization of two QTLs that increase phosphorus uptake of rice (*Oryza sativa* L.) under phosphorus deficiency. Plant Soil 237:275–286

Wissuwa M, Wegner J, Ae N, Yano M (2002) Substitution mapping of Pup1: a major QTL increasing phosphorus uptake of rice from a phosphorus-deficient soil. Theor Appl Genet 105:890–897

Wissuwa M, Gamat G, Ismail AM (2005) Is root growth under phosphorus deficiency affected by source or sink limitations? J Exp Bot 56:1943–1950

Yan JQ, Zhu J, He CX, Benmoussa M, Wu P (1998) Quantitative trait loci analysis for the developmental behavior of tiller number in rice (*Oryza sativa* L.). Theor Appl Genet 97:267–274

Yano M (2001) Genetic and molecular dissection of naturally occurring variation. Curr Opin Plant Biol 4:130–135

Yi K, Wu Z, Zhou J, Du L, Guo L, Wu Y, Wu P (2005) OsPTF1, a novel transcription factor involved in tolerance to phosphate starvation in rice. Plant Physiol 138:2087–2096

Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice, 3rd edn. IRRI, Manila

Zar JH (1999) Biostatistical Analysis, 4th edn. Prentice Hall, Upper Saddle River