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Substitution mapping of *Pup1*: a major QTL increasing phosphorus uptake of rice from a phosphorus-deficient soil

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Abstract A major QTL for P uptake had previously been mapped to a 13-cM marker interval on the long arm of chromosome 12. To map that major QTL with higher precision and certainty, a secondary mapping population was developed by backcrossing a near-isogenic line containing the QTL from the donor parent to the recurrent parent of low P uptake. Two different mapping strategies have been followed in this study. A conventional QTL mapping approach was based on individual F_2 RFLP data and the phenotypic evaluation of family means in the F_3. The second strategy employed a substitution-mapping approach. Phenotypic and marker data were obtained for 160 F_3 individuals of six highly informative families that differed in the size of donor chromosomal segments in the region of the putative QTL. QTL mapping showed that close to 80% of the variation between families was due to a single QTL, hereafter referred to as *Pup1* (Phosphorus uptake 1). *Pup1* was placed in a 3-cM interval flanked by markers S14025 and S13126, which is within 1 cM of the position identified in the original QTL mapping experiment. Other chromosomal regions and epistatic effects were not significant. Substitution mapping revealed that *Pup1* co-segregated with marker S13126 and that the flanking markers, S14025 and S13752, were outside the interval containing *Pup1*. The two mapping strategies therefore yielded almost identical results and, in combining the advantages of both, *Pup1* could be mapped with high certainty. The QTL mapping appoach showed that the phenotypic variation between families was due to only one QTL without any additional epistacic interactions, whereas the advantage of substitution mapping was to place clearly defined borders around the QTL.

Keywords Phosphorus deficiency · Near-isogenic line · Substitution mapping · Marker-assisted selection · *Oryza sativa*

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Introduction

Rice (*Oryza sativa* L.) yields are often limited by the low availability of phosphorus (P) in a variety of soils typically found in the tropics (Sanchez and Salinas 1981). The lack of locally available P sources and the high cost of importing and transporting P fertilizers frequently prevent resource-poor farmers in developing countries from applying P to their deficient fields (Sanchez and Salinas 1981). To develop rice cultivars with an improved tolerance to P-deficiency may therefore be a cost-effective solution to this problem.

Quantitative trait loci (QTLs) for P deficiency tolerance had been identified in a rice population derived from a cross of the intolerant *japonica* cultivar 'Nipponbare' with the tolerant *indica* landrace 'Kasalath' (Wissuwa et al. 1998). It was found that tolerance to P-deficiency was largely caused by genotypic differences in P uptake and that internal P-use efficiency had a negligible effect. A major QTL for P uptake was mapped to a 13.2-cM interval on the long arm of chromosome 12 flanked by markers C443-G2140. The position was estimated to be at 54.5 cM, a 3-cM distance from marker C443. Additional minor QTLs were found on chromosomes 2, 6, and 10. The first evidence supporting the presence of a major QTL for P deficiency tolerance came from a study by Ni et al. (1998). Using a different mapping population and screening method, these authors also identified a major QTL on chromosome 12. It remained unclear, however, whether that QTL affected P-uptake or internal P-use efficiency.

For further studies, a near-isogenic line (NIL) was developed by three backcrosses to Nipponbare. This near-isogenic line (NIL-C443) is genetically 91% identical to Nipponbare but carries a 50-cM Kasalath segment on chromosome 12 that includes the interval C443–G2140 containing the putative QTL. The effect of this major QTL has repeatedly been confirmed both in field experiments (Wissuwa and Ae 2001a) and in more detailed pot experiments (Wissuwa and Ae 2001b). NIL-C443 had about three-times the P uptake of Nipponbare when grown on P-deficient soil whereas both genotypes did not differ on soil supplied with an adequate amount of P. The effect of QTL C443 is therefore specific to P deficiency.

For the practical application of QTLs in marker-assisted selection (MAS) or for map-based cloning, the position of a QTL needs to be known with high reliability and precision (Paterson et al. 1990; Yano and Sasaki 1997; Yano et al. 2000). That requirement may be impossible to meet in primary QTL mapping experiments because typical mapping populations segregate for multiple genetic factors on the whole genome simultaneously (Yamamoto et al. 1998). Genetic parameters of each QTL are thus affected by the segregation of other QTLs. Currently QTL C443 is mapped to a 13.2-cM interval and the confirmation of QTL C443 was based on a NIL containing a 50-cM Kasalath segment. Such relatively low resolution is insufficient in MAS because an interval of 13 cM may contain a number of undesirable genes; particularly since Kasalath, the donor of QTL C443, is a landrace of low overall agronomic value. Furthermore, it is possible that a QTL mapped to a large interval corresponds to a cluster of genes, each with relatively small effects, rather than to a single locus. That would complicate attempts to transfer the QTL into elite breeding material. The identification and subsequent confirmation of our major QTL for P uptake therefore only represent two important first steps that need to be augmented by more precise mapping of QTL C443 before that QTL can be used in plant breeding.

Secondary mapping populations developed by backcrossing NILs to their recurrent parents are perfectly suited for fine mapping of QTLs (Yano 2001) because most genetic factors not related to the QTL no longer segregate. This concept has successfully been used to fine-map QTLs in maize (Dorweiler et al. 1993), tomato (Alpert and Tanksley 1996) and rice (Yamamoto et al. 1998). Their results showed that some QTLs could be treated as single Mendelian factors that are most likely due to a single gene.

We are following a similar approach to fine map QTL C443 in this study. A secondary mapping population was developed by backcrossing NIL-C443 to Nipponbare. At the 50-cM Kasalath segment all markers of the most-recent rice linkage map published by the rice genome project of Japan (http://rgp.dna.affrc.go.jp/Publicdata.html) were used in genotyping. Selected F_2 families were evaluated in a highly P-deficient field plot. In addition to a standard QTL analysis, we performed substitution mapping (Paterson et al. 1990) on a sub-set of lines that differed in the size and position of Kasalath chromosomal segments.

Materials and methods

Plant material

NIL-C443 had been developed by two additional backcrosses of a selected BC_1F_2 line from the primary mapping population to Nipponbare. Genotypic monitoring to maintain Kasalath alleles at QTL C443, as well as selection against Kasalath alleles at unrelated loci, was done using 118 RFLP markers evenly distributed throughout the rice genome. In addition to the 50-cM Kasalath segment on chromosome 12, NIL-C443 contains small Kasalath segments on chromosomes 1, 8, and 10 that had not been linked to P-uptake in the primary QTL analysis (see Fig. 3). The secondary mapping population used to fine-map the major QTL for P-uptake was developed by backcrossing NIL-C443 to Nipponbare. One hundred and fifty F_2 plants of that cross were genotyped using 42 RFLP markers, and a sub-set of 50 F_2 families was selected for phenotypic evaluation. This reduction allowed us to increase the number of replications to overcome the potentially high environmental variation for a trait like P uptake. The selection of F_2 families was based on their genotype for the Kasalath segment on chromosome 12. Thirty families were either homozygous Nipponbare or Kasalath on part of the segment and heterozygous on the remaining part; ten families were completely heterozygous; and five families each were completely homozygous for either Nipponbare or Kasalath.

Phenotypic evaluation

Phenotyping for P uptake was done using F_2-derived families in the F_3. The screening experiment was conducted in a field plot that had not received P fertilizer for 30 years. The soil type is a volcanic ash soil (Humic haplic andosol) with high P-fixing capacity. The predominant inorganic P forms are aluminum and iron-bound P; the more plant-available Ca-P was only detected in traces (Otani and Ae 1996). Soil from this field plot had also been used to detect and confirm P uptake QTLs. The 50 F_2 families were sown on May 10, 2000, in microplots (three rows of five plants) with five replications. Individual plants of Nipponbare and NIL-C443 were grown together with the mapping population as a control. After a growth period of 150 days, the number of tillers was recorded for all plants and used as an indirect estimate for their P uptake. Five plants of each tiller number class were sampled and their P-content analyzed to confirm that tiller number and P uptake are closely related.

In addition to using family mean values in mapping the major QTL for P uptake, we performed a second analysis using individual F_3 plants of six highly informative families. After recording their tiller number, DNA was extracted from 110 plants that clearly showed the NIL-C443 phenotype (two or more tillers). DNA of plants with a Nipponbare phenotype could not be extracted directly because P deficiency had strongly inhibited plant growth. Those plants therefore had to be dug out of the field and re-planted into pots containing soil well fertilized with P. After an additional growth period of 5 weeks in a heated greenhouse, sufficient new biomass had developed for DNA extraction. Only 50 plants of the Nipponbare phenotype were selected because of these complications. RFLP analysis of the 160 F_3 plants were performed with the same markers used in the F_2.

RFLP linkage mapping, QTL analysis, and substitution mapping

The 42 RFLP markers used in genotyping were selected from the high-density map of 3,267 RFLP markers published on the

web site of the Rice Genome Project, Japan (http://rgp.dna.affrc.go.jp/Publicdata.html). Twenty two markers were located on chromosomes 12, with 16 markers spanning the Kasalath segment between 30.0 and 72.5 cM. The remaining markers covered Kasalath segments on chromosomes 1 (eight markers), 8 (four markers) and 10 (eight markers). A linkage map for the 16 markers spanning the Kasalath segment on chromosome 12 was constructed based on marker data of 150 F_2 plants. Linkage analysis was done using the MAPMAKER program (Lander et al. 1987).

QTL analysis was performed using phenotypic data from 50 F_2 families. Computations were done with the software package PLABQTL (Utz and Melchinger 1996), which uses a multiple regression approach as suggested by Haley and Knott (1992). In a first step, simple interval mapping was performed and cofactors selected. For cofactor selection, F-to-enter and F-to-drop thresholds were set at 6.0 to avoid selecting multiple markers linked to one QTL as cofactors. Using these cofactors to reduce the residual variation, QTLs were detected using the composite interval mapping (CIM) method proposed by Zeng (1994). A further run was conducted with all markers on chromosome 12 selected as cofactors in order to detect multiple QTLs on a chromosome with greater resolution, as suggested by Utz and Melchinger (1996). A LOD score >2.50 was considered significant for QTL detection.

Furthermore, we attempted to detect the exact QTL position by a substitution mapping approach. Six highly informative families with a total of 160 individual F_3 plants were selected. They had shown one or two recombination events in the 50-cM Kasalath segment on chromosome 12 and thus differed in the length and position of the remaining Kasalath portion. Within each family the homozygous Kasalath or Nipponbare genotypes, as well as heterozygotes, were represented by several individual plants. The QTL position was analyzed by examining graphical genotypes of families in combination with their phenotypic values. Conventional QTL mapping was also done on the set of 160 individual F_3 plants using the same method described before.

Results

Evaluation of phenotypes

Relative tiller number (tiller number under P-deficiency relative to non-stress tiller number) has been used as an indirect estimate for P uptake (Ni et al. 1998). Employing a relative parameter allows for comparisons in stress response between a variety of diverse genotypes without confounding effects due to substantial differences in tillering ability. In this study, however, the parents used for the development of the mapping population was a pair of highly similar near-isogenic lines (Nipponbare and NIL-C443) that only differ in performance under P-deficient conditions (Wissuwa and Ae 2001b). A sub-set of F_3 lines from the secondary mapping population also showed no variation in tiller number when grown with added P fertilizer (unpublished data). Without variation under optimum P supply, the relative tiller number entirely depends on the number of tillers produced under P deficiency. We therefore used absolute, rather than relative, tiller number as an indirect estimate for P uptake in this study. The tight correlation between P uptake and the number of tillers observed in this field experiment (Fig. 1) confirms the validity of tiller number as a reliable indirect estimate for P uptake under P deficiency.

Both Nipponbare and NIL-C443 were included as checks in the fine mapping experiment and the number

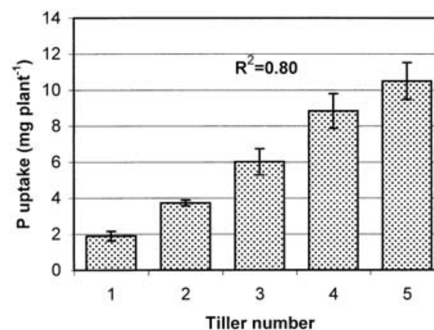


Fig. 1 Relationship between tiller number and phosphorus uptake (means and standard errors of five plants per tiller number class)

of tillers produced followed previously observed patterns. All Nipponbare check plants developed only one tiller (Fig. 2). The phenotypic class 'one tiller' could hence be used to identify plants of the Nipponbare genotype. NIL-C443 showed some variation around a mean of 2.5 tillers per plant. The Kasalath genotype therefore would be represented by the phenotypic class 'two or more tillers'. Using these classes, 8 out of 53 NIL-C443 plants would have been misclassified as having the Nipponbare phenotype. Since NIL-C443 is a fixed line that no longer segregates, this error in classification of about 15% would represent the experimental error that is caused by environmental variation (soil P fertility). Based on these results the phenotypic class 'two or more tillers' will reliably identify only plants of the Kasalath genotype, whereas the class 'one tiller' is expected to also include some outliers of the Kasalath genotype in addition to Nipponbare genotypes.

QTL mapping of *Pup1*

Map positions of markers on the Kasalath segment on chromosome 12 are shown in Fig. 3. This map is derived from segregation patterns of 150 F_2 individuals of the backcross of NIL-C443 to Nipponbare. Marker orders are identical to the high density map published by the Rice Genome Project (RGP), Japan (http://rgp.dna.affrc.go.jp/Publicdata.html), and distances between markers show a high degree of similarity with few exceptions.

Using F_2 family means for fine-mapping showed that 78.8% of the variation between families could be explained by one QTL on the long arm of chromosome 12 (Table 1). This major QTL is placed in the marker interval flanked by S14025 and S13126, and the precise location is estimated to be at a 1.3-cM distance from marker S14025. We hereafter use the designation *Pup1* (*P*hosphorus *up*take 1) for this major QTL. The QTL analysis furthermore indicated that *Pup1* was due to additive effects whereas dominance effects were not significant.

In addition to the large Kasalath segment on chromosome 12, NIL-C443 contains several other Kasalath segments on chromosomes 1, 8 and 10 (Fig. 3). None of

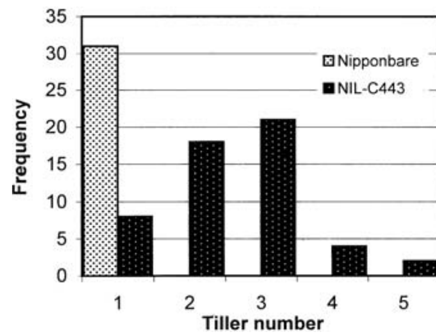


Fig. 2 Frequency distribution of Nipponbare and NIL-C443 for tiller number. Both genotypes had been grown as control plants together with the mapping population

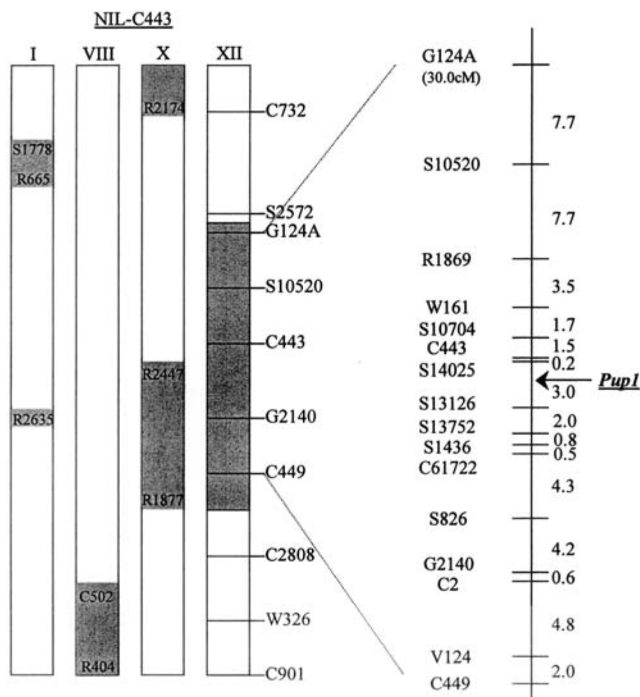


Fig. 3 Graphical genotype of NIL-C443 showing Kasalath segments on chromosomes 1, 8, 10 and 12, and linkage map of the Kasalath segment on chromosome 12 based on marker data of 150 F_2 plants of the secondary QTL-mapping population

these additional Kasalath segments had any effect on P uptake. Using a population of 160 individual F_3 plants instead of the family means for fine-mapping, placed *Pup1* in the same marker interval but closer to marker S13126 (Table 1). The variation explained by *Pup1* decreased to 28.1%. This is an expected result because phenotypic data was collected on individual plants without replications. As for F_2 families, the additive effect explained a larger portion of the variation ($R^2 = 0.27$) than the dominance effect ($R^2 = 0.05$); however, both effects were significant for F_3 plants. In the original QTL mapping experiment *Pup1* was placed in the marker interval C443–G2140 at a 3-cM distance from C443 (Wissuwa et al. 1998). The linkage map of Kurata et al. (1994) had been used in that experiment. Since map positions on chromosome 12 have been reversed on more recent maps, a 3-cM distance from C443 would correspond to a position of 54.5 cM on the current linkage map. This shows a high level of agreement with the present results.

The frequency distribution for the tiller number of F_2 families is shown in Fig. 4a. Marker S13126 was used for genotypic classification. Nipponbare genotypes produced an average of 1.07 tillers, heterozygote families were intermediate with a mean of 1.66 tillers and Kasalath genotypes produced 2.18 tillers. Due to the large number of replications (75 individuals per family), it was possible to quite clearly separate the homozygous classes while heterozygote families overlapped with both.

Homozygote F_3 individuals showed a frequency distribution similar to the one observed for the Nipponbare and NIL-C443 control plants shown in Fig. 2. Only one out of 30 Nipponbare genotypes had more than one tiller (Fig. 4b). Tiller numbers of Kasalath genotypes almost followed a normal distribution, ranging from one to five tillers with a mean of 2.57. Five out of 46 Kasalath genotypes (11%) were in the Nipponbare phenotypic class (one tiller). This was most likely due to environmental variation because we had observed a similar error in classification for NIL-C443 control plants (Fig. 2). Heterozygous F_3 individuals were not normally distributed but showed a concentration between one to three tillers with a mean of 2.13. This was above the mid-parent value of 1.83.

Table 1 Mapping of *Pup1*, a mayor QTL for P uptake on chromosome 12

Marker interval[a]	Position (cM)	LOD	R^2	Additive effect	Dominance effect
	Mapping based on F_2 derived families in F_3 ($n = 50$)				
<u>S14025</u>-S13126	53.6	16.6	78.9	0.56**	0.05ns
	Mapping based on individual F_3 plants ($n = 160$)				
S14025-<u>S13126</u>	54.8	11.5	28.1	0.69**	0.38*
	Primary QTL mapping (Wissuwa et al. 1998)				
<u>C443</u>-G2140	54.5[b]	10.7	27.9	–	–

[a] Nearest marker is underlined
[b] Map position on the current RFLP map where marker orders have been reversed in comparison to the linkage map (Kurata et al. 1994) used for QTL detection

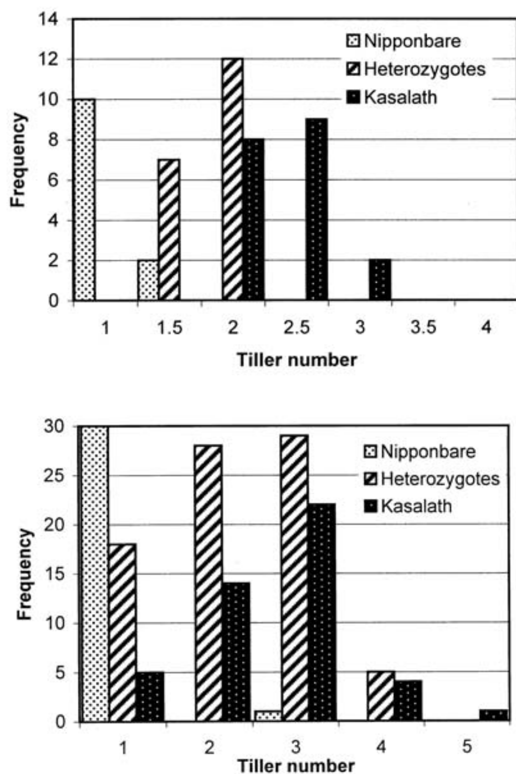
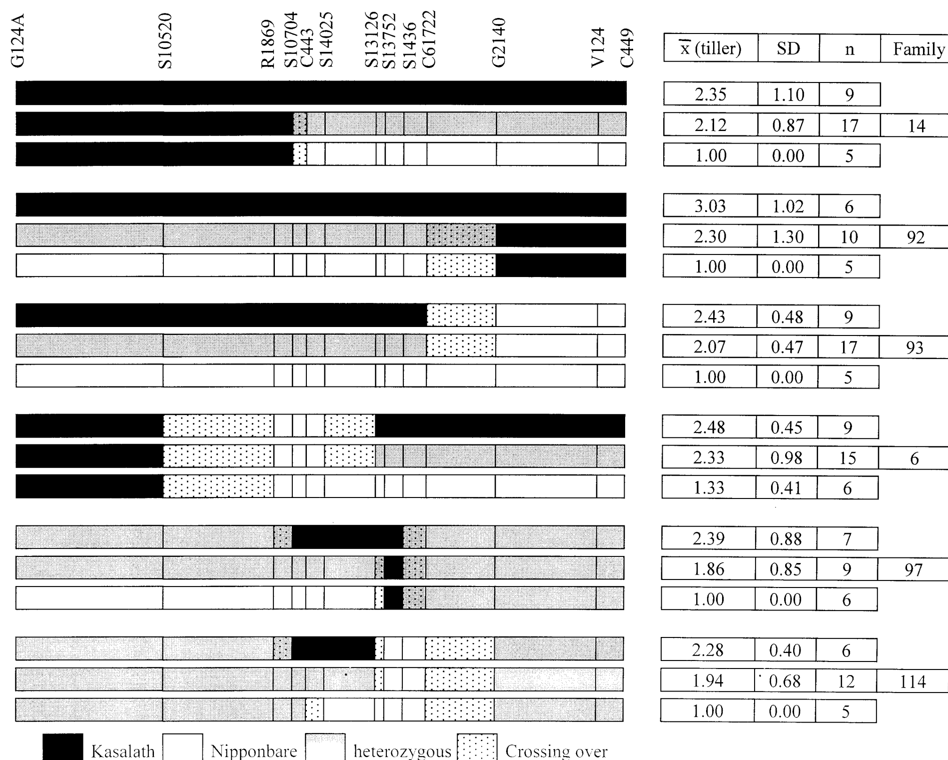


Fig. 4 Frequency distribution for tiller number under P deficiency for F_2 family means (Fig. 4a) and for 160 selected F_3 individuals of six F_2 families (Fig. 4b)

Substitution mapping of *Pup1*

Graphical genotypes at the Kasalath segment on chromosome 12 are shown for six highly informative families with a total of 160 individual F_3 plants in Fig. 5. Families differed in which of the five Kasalath segment on chromosomes 1, 8 and 10 they carried (see Fig. 3) but all plants within a family contained the same Kasalath segment. Variation within families was therefore only caused by genotypic variation on chromosome 12. The position of *Pup1* can be inferred from a step-wise exclusion process. From genotypes of family 14, it can be deduced that *Pup1* is located in the interval S10704–C449 because the Nipponbare genotype at that interval clearly produces the Nipponbare phenotype. Families 92 and 93 can be used to shorten the interval containing *Pup1* to S10704–G2140 because they show that genotypic variation outside that interval is not related to variation in tiller number. All lines of family 6 contain a Nipponbare insert between markers R1869–S14025 yet show the familiar phenotypic segregation (with the exception that one of the Nipponbare genotypes had three tillers). This would imply that S14025, identified as a flanking marker in the QTL analysis, would lie outside the interval containing *Pup1*. Families 97 and 114 finally lead to the exclusion of the interval S13752–G2140 (–C449). It can thus be concluded that marker S13126 is the only one that co-segregates with *Pup1*.



$\bar{x}$ (tiller)	SD	n	Family
2.35	1.10	9	
2.12	0.87	17	14
1.00	0.00	5	
3.03	1.02	6	
2.30	1.30	10	92
1.00	0.00	5	
2.43	0.48	9	
2.07	0.47	17	93
1.00	0.00	5	
2.48	0.45	9	
2.33	0.98	15	6
1.33	0.41	6	
2.39	0.88	7	
1.86	0.85	9	97
1.00	0.00	6	
2.28	0.40	6	
1.94	0.68	12	114
1.00	0.00	5	

Fig. 5 Graphical genotypes for the Kasalath segment on chromosome 12 of 160 selected F_3 individuals of six F_2 families, as well as the means and standard deviations for tiller numbers of genotypic classes within families

Discussion

Mapping of *Pup1*

Following the identification of a major QTL for P-uptake (*Pup1*) in the primary mapping experiment, the effect of that QTL had been confirmed using a near-isogenic line. That NIL-C443 had about three times the P uptake of Nipponbare (Wissuwa and Ae 2001a, b) proved that *Pup1* continued to show its effect in isolation from much of the donor genome. Using NILs, however, poses the risk that small donor chromosomal segments (relics) persist and that their potential effect can not be accounted for (Paterson et al. 1990). Such relics will be randomized in a segregating population like the one developed from a backcross of NIL-C443 with Nipponbare and can therefore be factored out in secondary mapping experiments. Our results showed that none of the relics carried by NIL-C443 had any effect on P-uptake. Instead, close to 80% of the variation for tiller number between F_2 families was explained by genotypic variation at the *Pup1* locus. Epistatic interactions between *Pup1* and the remaining genome were also not significant. The number of F_2 families had been limited to 50 in favour of a high number of replications per family (75) whereas a larger number of F_3 plants (160) was used without replications. That both sets of data yielded almost identical results indicated that the small population size in the F_2, or the lack of replications in the F_3, did not limit the validity of the QTL analysis. The large number af replications in the F_2, however, had the positive effect of increasing the portion of the variation explained by the genetic model to almost 80% (compared to 28% in the F_3).

Two different approaches have been used in the present study to determine the exact location of *Pup1*. QTL analysis placed *Pup1* in a 3-cM interval flanked by markers S14025 and S13126, regardless of whether the F_2 family means or the individual F_3 plant data were used. The support interval identified in QTL mapping also included the interval S13126–S13752. Substitution mapping showed that *Pup1* only co-segregates with marker S13126. The interval would thus be bordered by markers S14025–S13752 with both flanking markers lying outside the interval actually containing *Pup1*. Both mapping approaches therefore yielded almost identical results if the support interval is taken into consideration. The advantage of substitution mapping lies in the ability to clearly visualize borders of an interval containing the QTL, whereas conventional QTL mapping assigns positions based on probabilities. Those probabilities depend on distances between markers, as the current example clearly shows. With 3.3 cM the interval S14025–S13126 is much larger and thus more likely to contain *Pup1* than the interval S13126–S13752 (0.8 cM). However, substitution mapping indicated that *Pup1* may also lie in the smaller of the two intervals.

Recombination events in the interval S14025–S13126 of family 6 and in the interval S13126–S13752 of families 97 and 114 (Fig. 5) could potentially be used for higher resolution mapping. However, no additional markers have been mapped in these intervals at this stage and therefore it was not possible to use the segregation patterns of families 6, 97 and 114 to further narrow down the exact position of *Pup1*. The initial QTL mapping experiment placed *Pup1* within a 1-cM distance of the present position (Table 1). That *Pup1* is a major QTL was certainly the main factor responsible for the high level of agreement between mapping experiments. However, Yamamoto et al. (1998) were able to show that even minor QTLs had initially been mapped with high precision.

Whether a QTL can be treated as a single Mendelian factor (possibly caused by a single gene) or is the result of a cluster of minor genes is a question that has been raised earlier. That only one QTL has subsequently been mapped on chromosome 12, and that this QTL explained close to 80% of the variation for tiller number, suggests that *Pup1* may be treated as a single Mendelian factor. However, with 3 cM the interval containing *Pup1* is large enough to contain several genes. It can thus not be ruled out that *Pup1* is caused by several closely linked genes.

Application of current results in marker-assisted selection (MAS)

Despite its high tolerance to P deficiency, the direct use of Kasalath as a cultivar can not be recommended because of several undesirable attributes. Kasalath is a tall variety susceptible to lodging and its small grains shatter easily. The preferable approach would be to transfer *Pup1* into plant material of higher overall agronomic value using Kasalath as the donor. This can be achieved by traditional backcross breeding procedures but marker-assisted selection would greatly facilitate this process (Melchinger 1990; Hospital and Charcosset 1997). Selection for markers linked to *Pup1* rather than for phenotype (foreground selection) is particularly advantageous for a trait like P uptake under P deficiency, that is only expressed in certain environments and is affected by environmental variation to a high degree (see Figs. 2 and 4). Employing MAS would thus reduce time and labor-intensive field evaluations on P-deficient soil to a minimum during the backcross procedure. Selection for individuals that are homozygous for the recurrent parent allele at a large number of marker loci covering the entire genome (background selection) furthermore reduces the number of generations needed for the recovery of the recurrent parent genome at unrelated loci (Hospital and Charcosset 1997). Frisch et al. (1999) estimated that four backcross generations, aided by MAS employing small populations and a limited number of marker points, would suffice to transfer a single target allele. This would save two generations compared to traditional backcross methods. With larger populations and marker points, savings could increase to three generations.

Pup1 has been mapped with very high certainty to a relatively small marker interval in this study. The flank-

ing markers S14025 and S13126 (S13752) could thus be used by rice breeders for marker-assisted introgression of *Pup1* into elite breeding material to combine their positive attributes with high tolerance to P deficiency. With 3–4 cM the interval is small enough to reduce linkage drag, the introgression of undesirable traits together with the target allele, to a level that may be acceptable for plant breeders (Frisch et al. 1999). For the purpose of map-based cloning, however, a 3–4-cM interval remains too large. Additional tightly linked markers will have to be developed for high-resolution mapping of *Pup1* to pursue the final goal of cloning the gene(s) at *Pup1*.

Using near-isogenic lines in physiological studies

Near-isogenic lines are not only powerful tools in genetic studies but also permit the examination of physiological processes linked to a gene because they reduce the variation unrelated to the target trait. We have used NIL-C443 in comparison to Nipponbare and to one additional NIL that contained the same Kasalath segments on chromosomes 1, 8 and 10, but lacked the insert on chromosome 12, to characterize the major P-uptake QTL (Wissuwa and Ae 1999). That study showed that only chromosome 12 contributed to differences in P-uptake; a result that has been confirmed in the present study. We were, however, not able to identify the physiological mechanism responsible for the difference between genotypes in P uptake. Subsequent experiments were designed to determine whether high P uptake of NIL-C443 was caused by higher root growth or by more efficient P uptake per unit root size (root efficiency). Results of that study showed that seedling root growth rates of NIL-C443 were enhanced under P deficiency (Wissuwa and Ae 2001b). But NIL-C443 maintained a higher P uptake even when differences in seedling root growth rates were eliminated. This would imply that *Pup1* affects both root growth and root efficiency. The assumption of one QTL, one gene or one causal mechanism may thus not be correct. *Pup1* may consist of a cluster of genes each affecting different mechanisms, or may be due to one stress response/signalling gene that up or down regulates a number of other genes that may not even be located in the vicinity of the QTL.

But it is also feasible that *Pup1* only affects one causal mechanism such as root efficiency and that higher seedling root growth is of secondary importance. Since NIL-C443 is only 91% similar to Nipponbare with 9% of its alleles being from Kasalath, secondary effects could have been caused by allelic differences at loci outside the interval containing *Pup1*. The only way to solve these questions may be a genetic approach. Once the gene(s) at the QTL has(ve) been cloned, their function can be inferred from comparisons to gene-databases. Until that has been accomplished, each potential mechanism should be tested with several NILs that segregate for *Pup1* as well as for unrelated Kasalath alleles (relics). Only traits that co-segregate with the QTL and with high P uptake are likely to be directly involved in the tolerance mechanism. New NILs can be developed as a by product of fine-mapping. Families used in substitution mapping (Fig. 5) would offer an excellent starting point, as NILs that differ in the size and position of Kasalath segments can be extracted directly or after one additional generation. Using a whole set of NILs in physiological experiments should ultimately yield more detailed insights into the physiology of phosphorus uptake.

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