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ORIGINAL PAPER

Development and application of gene-based markers for the major rice QTL *Phosphorus uptake 1*

Joong Hyoun Chin · Xiaochun Lu · Stephan M. Haefele · Rico Gamuyao · Abdelbagi Ismail · Matthias Wissuwa · Sigrid Heuer

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Abstract Marker-assisted breeding is a very useful tool for breeders but still lags behind its potential because information on the effect of quantitative trait loci (QTLs) in different genetic backgrounds and ideal molecular markers are unavailable. Here, we report on some first steps toward the validation and application of the major rice QTL *Phosphate uptake 1* (*Pup1*) that confers tolerance of phosphorus (P) deficiency in rice (*Oryza sativa* L.). Based on the *Pup1* genomic sequence of the tolerant donor variety Kasalath that recently became available, markers were designed that target (1) putative genes that are partially conserved in the Nipponbare reference genome and (2) Kasalath-specific genes that are located in a large insertion-deletion (INDEL) region that is absent in Nipponbare. Testing these markers in 159 diverse rice accessions confirmed their diagnostic value across genotypes and showed that *Pup1* is present in more than 50% of rice accessions adapted to stress-prone environments, whereas it was detected in only about 10% of the analyzed irrigated/lowland varieties. Furthermore, the *Pup1* locus was detected in more than 80% of the analyzed drought-tolerant rice breeding lines, suggesting that breeders are unknowingly selecting for *Pup1*. A hydroponics experiment revealed genotypic differences in the response to P deficiency between upland and irrigated varieties but confirmed that root elongation is independent of *Pup1*. Contrasting *Pup1* near-isogenic lines (NILs) were subsequently grown in two different P-deficient soils and environments. Under the applied aerobic growth conditions, NILs with the *Pup1* locus maintained significantly higher grain weight plant^{-1} under P deprivation in comparison with intolerant sister lines without *Pup1*. Overall, the data provide evidence that *Pup1* has the potential to improve yield in P-deficient and/or drought-prone environments and in diverse genetic backgrounds.

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J. H. Chin · S. M. Haefele · R. Gamuyao · S. Heuer (✉)
International Rice Research Institute (IRRI), Plant Breeding, Genetics, and Biotechnology Division (PBGB),
DAPO Box 7777, Metro Manila, Philippines
e-mail: s.heuer@cgiar.org

Present Address:
X. Lu
College of Life Science, Zhejiang University,
Zeijang Campus, 29, 310058 Hangzhou, China

A. Ismail
International Rice Research Institute (IRRI),
Crop and Environmental Sciences Division (CESD),
DAPO Box 7777, Metro Manila, Philippines

M. Wissuwa
Japan International Research Center for Agricultural Sciences (JIRCAS), 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan

Introduction

An increasing number of quantitative trait loci (QTLs) and candidate genes associated with plant responses to abiotic and biotic stresses are being reported in rice and other important crops. Several recently published review papers provide a comprehensive overview of the efforts of breeders, geneticists, and molecular biologists that are under way worldwide (Xu and Crouch 2008; Mackill 2008; Collins et al. 2008). However, so far, very few QTLs are actively targeted in breeding programs since most QTLs

are not validated in different genetic backgrounds and environments, and suitable molecular markers are not available. The development of simple sequence repeat (SSR) markers, and more recently the development of single nucleotide polymorphism (SNP) markers, will in the near future provide breeders with a low-cost, high-throughput technology that will facilitate the screening of large breeding populations for target QTLs and candidate genes (Collard and Mackill 2008; Zhao et al. 2009). The potential impact of marker-assisted selection (MAS) has recently been demonstrated by developing submergence-tolerant rice varieties. The major QTL for submergence tolerance, *Submergence 1* (*Sub1*) located on chromosome 9, had been sequenced in the tolerant donor parent and the *SUB1A* gene was identified as the major determinant of submergence tolerance (Xu et al. 2006). Using a combination of gene-based foreground markers and closely flanking SSR markers in conjunction with an optimized phenotyping system, the *Sub1* locus was introgressed into several widely grown, well-adapted Asian rice varieties (Neeraja et al. 2007; Septiningsih et al. 2009). The genetic background of the recipient parent was then restored by repeated backcrossing (BC). The final products of this approach are submergence-tolerant rice varieties that are otherwise indistinguishable from the original variety, and desirable traits, e.g., good agronomic performance and grain quality, remain unchanged. The first two Sub1 varieties have very recently been released in India (Swarna-Sub1) and the Philippines (IR64-*Sub1*) and are already grown in farmers' fields.

One of the major findings within the *Sub1* study was that the actual tolerance gene (*SUB1A*) is not present in the Nipponbare reference genome and was identified only after the *Sub1* genomic region was sequenced in the tolerant donor. A similar situation was found in the major QTL *Phosphate uptake 1* (*Pup1*). *Pup1* was reported to confer tolerance of phosphorus (P) deficiency under field conditions in Japan (Wissuwa et al. 1998; Wissuwa et al. 2002) and was independently mapped by Ni et al. (1998). Since physiological analyses of the tolerance-underlying mechanisms in near-isogenic lines (NILs) did not reveal any evidence as to how *Pup1* improves P uptake (Wissuwa 2005), the genomic region was sequenced in the tolerant donor parent Kasalath to identify the genes present in the region. Comparative genomic analyses subsequently revealed a highly complex genomic structure with overall little conservation between the Kasalath *Pup1* region (~280 kb) and the syntenic regions in the *japonica* (Nipponbare: ~150 kb), and *indica* (93–11: ~750 kb) reference genomes (Heuer et al. 2009). A large number of transposable elements (TEs, 45–54%) present in all three loci can at least partly explain the observed size differences, and this is causing considerable problems in the prediction of gene models (Heuer et al. 2009). Overall, only three out of the 68 predicted *Pup1* genes show a high degree of sequence similarity between Nipponbare and Kasalath, none of them obviously related to P uptake. This is in agreement with transcription profiling data showing that known P-responsive genes are not differentially regulated in *Pup1* NILs grown under P-deficient conditions compared to Nipponbare (Pariasca-Tanaka et al. 2009). Detailed analyses of the *Pup1* candidate genes and the generation of transgenic plants for gene validation are now ongoing (Heuer et al., unpublished).

The complexity of the *Pup1* genomic sequence and the fact that *Pup1* is likely not acting via known P uptake mechanisms has several implications for marker development and the development of a phenotyping system. For instance, the development of SNP markers is based on sequence differences of genes present in both respective parents used for the crosses. In the case of *Pup1*, only three to four out of the 68 predicted putative genes (including TEs) qualify for this approach since many *Pup1* genes are located in an insertion-deletion (INDEL) region that is specific to Kasalath (Heuer et al. 2009; this paper). It is therefore necessary to develop other types of markers in order to be able to analyze the entire locus. Indeed, a first set of *Pup*-specific SSR markers for recombinant selection has recently been published by Collard et al. (2006), but the authors failed to identify suitable SSR foreground markers diagnostic of *Pup1*.

Likewise, the development of a high-throughput phenotyping system is challenging as long as the function and precise action of *Pup1* is not entirely understood. The *Pup1* locus was mapped under rainfed conditions in a field with P-fixing volcanic soil in Japan. Subsequent experiments that confirmed the effect of *Pup1* in a set of NILs were conducted in greenhouse trials using the same soil (Wissuwa 2005). As a first step toward the application of *Pup1,* it is therefore essential to demonstrate that *Pup1* provides an advantage in other rice environments and in different genetic backgrounds. The potential benefit of *Pup1* is high since P deficiency is recognized as a major constraint to the production of rice and other crops. In high-input systems, the application of P fertilizer can correct low P content as was shown in Indonesia, where about 30% of lowland rice areas were considered P-deficient in the 1970s in contrast to only 17% today due to a regular application of P fertilizer (FAO 2005). However, P fixation in soils with a high content of free ferric oxides in the clay fraction and high aluminum (Al) concentration is a widespread problem and limits access of plants to P even if it is present in the soil. According to FAO Terrastat data (www.fao.org), P fixation occurs on 4% of the total land area in sub-Saharan Africa (SSA), on 5% within the Asia and Pacific region, and is affecting as much as 25% of the

total land area in Brazil alone. Countries in Asia with the highest percentage of total land area affected by P fixation are Laos (24%), Vietnam (15%), Myanmar (16%), and Thailand (11%), as well as China, Indonesia, and Japan (all 9%). A recently published soil constraint map overlaid with rainfed rice-growing areas showed that, in Asia, about 60% of rainfed rice is grown on soils that are affected by multiple stresses, including P deficiency (Haefele and Hijmans 2007). The development of rice varieties that can extract P from P-fixing soils and that have a higher P fertilizer use efficiency in combination with tolerance of other abiotic stresses (e.g., acidity, salinity, Al toxicity, drought) is therefore considered an important breeding goal (Ismail et al. 2007).

The main objectives of this study were therefore to (1) develop *Pup1*-specific markers that can be used for the development of *Pup1*-introgression lines, (2) determine the *Pup1* haplotype in a diverse set of rice accessions, and (3) validate the phenotypic effect of *Pup1*.

Materials and methods

Plant material

Seeds of 159 rice accessions were obtained from the International Rice Germplasm Collection (IRGC) of the International Rice Research Institute (IRRI) and from IRRI rice breeders. The contrasting *Pup1* NILs used in this study were developed from a Nipponbare × Kasalath population (Wissuwa et al. 2002; Wissuwa 2005). NIL14-4 and NIL6-4 carry the Kasalath *Pup1* introgression on Chromosome 12, whereas the *Pup1* locus is absent from the sister lines NIL14-6 and NIL6-3.

Information on the genetic background of the screened accessions (*indica, japonica, aus*; traditional or modern variety) and preferred cropping system (unfavorable rainfed, favorable lowland/irrigated) was derived from a literature review, Google™ searches, and personal communication with IRRI breeders. Details are given as supplementary data (Table S1; Fig. S1).

Phenotyping in hydroponics and P-deficient soils

For a hydroponics experiment, *Pup1* NILs and 13 additional genotypes were grown for 52 days in a greenhouse on a table about 50 cm below 100 W clear light bulbs (16 h light/8 h dark). Long-day conditions were applied to induce tillering and prevent early flowering in the *Pup1* NILs caused by the photosensitive Nipponbare background. Plants were grown in 50-L buckets filled with Yoshida nutrient solution (Yoshida et al. 1972) with modified P concentration (0 and 100 μM KH_2PO_4 for low P and high P, respectively). The pH was adjusted every 3 days and the solution was changed once a week.

For a soil experiment, the contrasting NILs 14-4 (+*Pup1*) and 14-6 (−*Pup1*) were grown in a controlled growth chamber (28°C/21°C; 15.5 h light/8.5 h dark; light intensity 0.421 MJ m^{-2} day^{-1}; relative humidity 70%) for 50 days before they were transferred to a greenhouse with natural (12–13 h) light conditions to induce reproductive growth. Plants were grown in 20-L buckets filled with 20 kg P-deficient topsoil collected from a farmer's field located in Kapatalan, Laguna, Philippines. The average P content of random soil samples from that site was 6.2 ± 0.42 mg kg^{-1} according to a Bray2 analysis conducted at the analytical service laboratory at IRRI. The soil was treated with Furadan® (Soriano and Reversat 2003) before the start of the experiment to control nematodes and other root pathogens. Nitrogen (N; 12.48 g urea; 2 splits: basal and at maximum tillering stage) and potassium (K; 3.20 g muriate of potash), and zinc (Zn; 0.64 g $ZnSO_4$) fertilizers were added to all pots. Phosphorus fertilizer (10.72 g single super phosphate, 15–17% P_2O_5) was added only to +P control pots. Three plants of *Pup1* NILs 14-4 and 14-6 each were grown in every pot to ensure similar growth conditions and the soil was kept aerobic but well watered at all times. At 46 days after sowing (DAS), three pots per treatment were harvested and six plants per NIL and treatment were analyzed. At harvest (115 DAS), 3–4 pots per treatment were harvested and 6–10 plants per NIL and treatment were analyzed.

An additional experiment was conducted in a greenhouse at JIRCAS, Tsukuba (Japan) during 2007 using Nipponbare and five NILs with contrasting *Pup1* haplotypes (+*Pup1*: NIL6-4, NIL14-4, NIL24-4; −*Pup1*: NIL14-6, NIL24-6; see Heuer et al. 2009). Plants were grown in 40-L buckets filled with P-deficient soil derived from a field in Tsukuba, Japan (Wissuwa and Ae 2001a) in three replicates without addition of P fertilizer or with a fertilizer dose equivalent of 50 kg P_2O_5 ha^{-1}. Both treatments received the equivalent of 70 kg ha^{-1} N and 50 kg ha^{-1} K_2O, and soil was kept well watered but aerobic. P content in shoots and seeds was determined by the phosphovanadate method (Hanson 1950) after digestion in a mixture of HNO_3, $HClO_4$, H_2SO_4 (3:1:1).

Genomic DNA extraction and molecular markers

Pup1-specific markers were designed based on the available *Pup1* sequence information (Heuer et al. 2009). The genomic sequence of the *Pup1* locus and flanking region is available under the accession number AB458444 at DDBJ. A BLASTn search with the genomic sequence of the predicted *Pup1* genes was conducted at TIGR, NCBI, and NIAS gene databases (http://blast.jcvi.org/euk-blast/index.cgi?project=osa1; http://blast.ncbi.nlm.nih.gov/Blast.cgi;

http://riceblast.dna.affrc.go.jp/) to identify similar genes from Nipponbare and other rice reference gene information. Several of the targeted *Pup1* genes are located in INDEL regions and are not found in the databases. Based on these sequence analyses, primers specifically amplifying the targeted Kasalath genes were designed using Primer3 v.0.4.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The specificity of primers was re-confirmed by TIGR and NCBI BLASTn searches. Primers were synthesized by SBS (Genetech Co. Ltd., Beijing, China). Out of all primer pairs tested (see text for details), nine markers were selected that were either dominant for the Kasalath allele (targeting genes in the INDEL region) or co-dominant (targeting genes partially conserved between Nipponbare and Kasalath). Primer sequences and details on the targeted *Pup1* genes are given in Table 1.

Leaf samples from rice seedlings for genomic DNA extraction were collected in Falcon tubes and immediately frozen in liquid nitrogen. Samples were stored at −80°C until DNA was extracted according to Pallotta et al. (2000). Standard PCR was carried out using a G-storm GS1 thermocycler (Applied Biosystem) with the following profile: 5 min 94°C, 30 cycles: 30 s 94°C, 45 s 55–60°C, 60 s 72°C, followed by 10 min at 72°C for a final extension. Genomic DNA (20–50 ng) was used as template in a total volume of 20 µl (5 pmol each primer, 2 µl PCR buffer [100 mM Tris–HCl, 400 mM KCl, 15 mM $MgCl_2$, pH 9.0], 1 µl of 10 mM dNTPs, and 0.5 unit *Taq* polymerase (SBS Genetech, Beijing, China). PCR products were size fractionated in 1.4% agarose gels and stained with either ethidium bromide or SyberSafe (Invitrogen, Oregon, USA).

Statistical analysis

Data were analyzed and graphically illustrated using Microsoft Excel software. Significant differences between data sets were determined according to Tukey's HSD test or one-tailed *t* test at significance level of 0.05.

Results

Development of *Pup1*-specific PCR-based molecular markers

The *Pup1* sequence was recently assembled from Kasalath BAC clones and 68 putative genes, including transposon and retro-transposon-related elements (TEs), were predicted (Heuer et al. 2009). The *Pup1* locus has a complex genetic structure and shows overall little sequence similarity to the syntenic region in the Nipponbare reference genome (Fig. 1a). In order to facilitate marker-assisted introgression of *Pup1* in breeding lines and to gain insight into the evolution and distribution of *Pup1* in rice germplasm, we have designed primer pairs throughout the Kasalath *Pup1* locus specifically targeting putative genes.

Table 1 Sequences of gene-specific and fine-mapping *Pup1* markers

Marker name[a]	Expected size (bp) Kas/Nip	Tm (°C)	Primer sequence
Pup1-K20	240/243	55	for 5′-TCAGGTGATGGGAATCATTG-3′
			rev 5′-TGTTCCAACCAAACAACCTG-3′
Pup1-K29	480/491	55	for 5′-CCATAGTAGCACAAGAAACCGACA-3′
			rev 5′-GCTTCAATGAGCCCAGATTACGAA-3′
Pup1-K41	382/null[b]	58	for 5′-TGATGAATCCATAGGACAGCGT-3′
			rev 5′-TCAGGTGGTGCTTCGTTGGTA-3′
Pup1-K42	918/null	58	for 5′-CCCGAGAGTTCATCAGAAGGA-3′
			rev 5′-AGTGAGTGGCGTTTGCGAT-3′
Pup1-K43	912/null	58	for 5′-AGGAGGATGAGCCTGAAGAGA-3′
			rev 5′-TCGCACTAACAGCAGCAGATT-3′
Pup1-K46	523/null	58	for 5′-TGAGATAGCCGTCAAGATGCT-3′
			rev 5′-AAGGACCACCATTCCATAGC-3′
Pup1-K48	847/null	58	for 5′-CAGCATTCAGCAAGACAACAG-3′
			rev 5′-ATCCGTGTGGAGCAACTCATC-3′
Pup1-K52	505/null	58	for 5′-ACCGTTCCCAACAGATTCCAT-3′
			rev 5′-CCCGTAATAGCAACAACCCAA-3′
Pup1-K59	550/null	58	for 5′-GGACACGGATTCAAGGAGGA-3′
			rev 5′-TGCTTTCCATTTGCGGCTC-3′
Ba76H14_7154	292/259	55	for 5′-GAAACGGGGTCAAATAAGC-3′
			rev 5′-GGGTTCGTCCAACAGGAGTA-3′

[a] The nomenclature of the markers corresponds to the gene identifiers published by Heuer et al. (2009)

[b] Dominant marker, no amplicon in Nipponbare

These primer pairs were initially tested in Nipponbare, Kasalath, and contrasting *Pup1* NILs, as well as in a set of diverse rice genotypes. Seven of the tested primer pairs amplified a DNA fragment in Kasalath and the *Pup1* NIL14-4 (+*Pup1*), but not in Nipponbare or the *Pup1* control sister line, NIL14-6 (−*Pup1*). These dominant markers are all located within an INDEL region, and they target seven putative *Pup1* genes, including transposon-related genes (Fig. 1a; Table 1). The INDEL region does not show significant sequence similarity to the Nipponbare and 93-11 syntenic regions on Chr. 12 or any other rice chromosome according to BLASTn and BLASTp analyses (Heuer et al. 2009) and is therefore highly specific for the Kasalath *Pup1* locus. Since none of the tested markers were co-dominant or diagnostic for the 5′-end of the *Pup1* region, a second set of primers was designed based on more detailed comparative sequence analyses targeting regions that are at least partially conserved between Kasalath and Nipponbare. Two markers targeting the partially conserved genes *PupK20-1* (dirigent-like gene) and *PupK29-1* (hypothetical protein) amplified DNA fragments of different size in *Pup1* and non-*Pup1* genotypes and were therefore included in the germplasm survey (Fig. 1; Table 1). The targeted genes are described in more detail by Heuer et al. (2009).

In addition to the foreground markers, the *Pup1*-flanking region was analyzed to identify markers suitable for recombinant selection. From a set of 31 tested SSR and STS markers (data not shown), 11 polymorphic markers located at the 5′ and the 3′ *Pup1*-flanking regions were identified (Figs. 1, 2). The markers RM28073 and RM28102 are located closest to the *Pup1* locus (at 14.95 and 15.91 Mb in the TIGR5 reference genome). The *Pup1* fine-mapping marker that defined the 3′-border of *Pup1* (Ba76H14_7154) was additionally included in the analysis (Heuer et al. 2009).

Germplasm survey with *Pup1* markers

To further validate the markers, a total of 159 rice accessions obtained from the International Rice Germplasm Collection and IRRI breeders were genotyped with the developed *Pup1*-specific dominant and co-dominant markers (Figs. 1b, 2). Flanking markers and markers located at a larger distance from *Pup1* on Chr. 12 were analyzed in a subset of 66 genotypes. The complete list of analyzed rice accessions is given in the supplementary data (Supplementary Table S1). Based on the Kasalath-allele frequency of the seven dominant markers, genotypes were divided into a Kasalath-like group (K-group, >50% Kasalath alleles) and a non-Kasalath group (N-group, <50% Kasalath alleles). According to this classification, 84 genotypes belong to the K-group and 75 genotypes to the N-group (Fig. 1; Table S1). Markers K42 and K52 were most diagnostic for *Pup1* since Kasalath alleles were detected in about 90% of the genotypes in the K-group but were never detected within the N-group. The markers K41, K43, and K46 are slightly less diagnostic since Kasalath alleles were represented in some (<20%) genotypes of the N-group. Among the dominant markers, K48 had the least diagnostic value for *Pup1* although the specificity of this primer pair for the Kasalath *Pup1* sequence was verified by BLASTn searches, as has been done for the other markers.

As expected, the markers located outside of *Pup1* on Chr. 12 were not diagnostic for *Pup1* since Kasalath and non-Kasalath alleles were equally present in the K- and N-groups within the subset of 52-77 genotypes that were analyzed with these markers (Figs. 1b, 2; and Supplementary Table S1). The data further showed that the marker Ba76H14_7154 that was used for fine mapping of the *Pup1* locus (Heuer et al. 2009) is largely monomorphic or absent in the analyzed genotypes and therefore not suitable for *Pup1* fine mapping using parents other than Kasalath and Nipponbare (Figs. 1b, 2, bottom panel). As illustrated in Fig. 2, for a representative set of 58 genotypes, the dominant markers can be used in an agarose-based detection system and are diagnostic in a wide range of diverse rice genotypes.

The germplasm survey showed that the *Pup1* locus was over-represented in genotypes that were developed for unfavorable, drought-prone environments (indicated by "+" in Fig. 2). The data show that 34 (85%) of the 40 genotypes that are considered drought tolerant based on screenings at IRRI (unpublished data) possess Kasalath alleles at all or most targeted genes (Fig. 2). Only one tolerant genotype (#53, IR70617-4B-B-19-2-3-1-1) did not possess the *Pup1* locus. Within the drought-intolerant group, only one genotype (#12, Jalmagna) possessed the tolerant *Pup1* haplotype, whereas all others showed the expected absence of Kasalath alleles. However, phenotypic data from Japan (Wissuwa et al., unpublished) showed good performance of Jalmagna and intermediate performance of IR70617-4B-B-19-2-3-1-1 under P-deficient upland conditions, which is in agreement with our *Pup1* genotypic data. When the analyzed genotypes were sorted according to their varietal group (*indica*, *japonica*, *aus*) and cropping system (upland, lowland/irrigated) it became evident that the *Pup1* locus is present in many of the analyzed upland varieties of both *indica* (56.2%) and *japonica* (50.7%) types, whereas it is largely absent from lowland and irrigated *japonica* and *indica*-type varieties (Fig. 3a; Supplementary Fig. S1 and Table S1). The data further showed that *Pup1* is present at a higher frequency in traditional varieties, whereas it was detected in only a few of the analyzed modern lowland/irrigated genotypes (Fig. 3b). In contrast, the *Pup1* locus has been conserved in

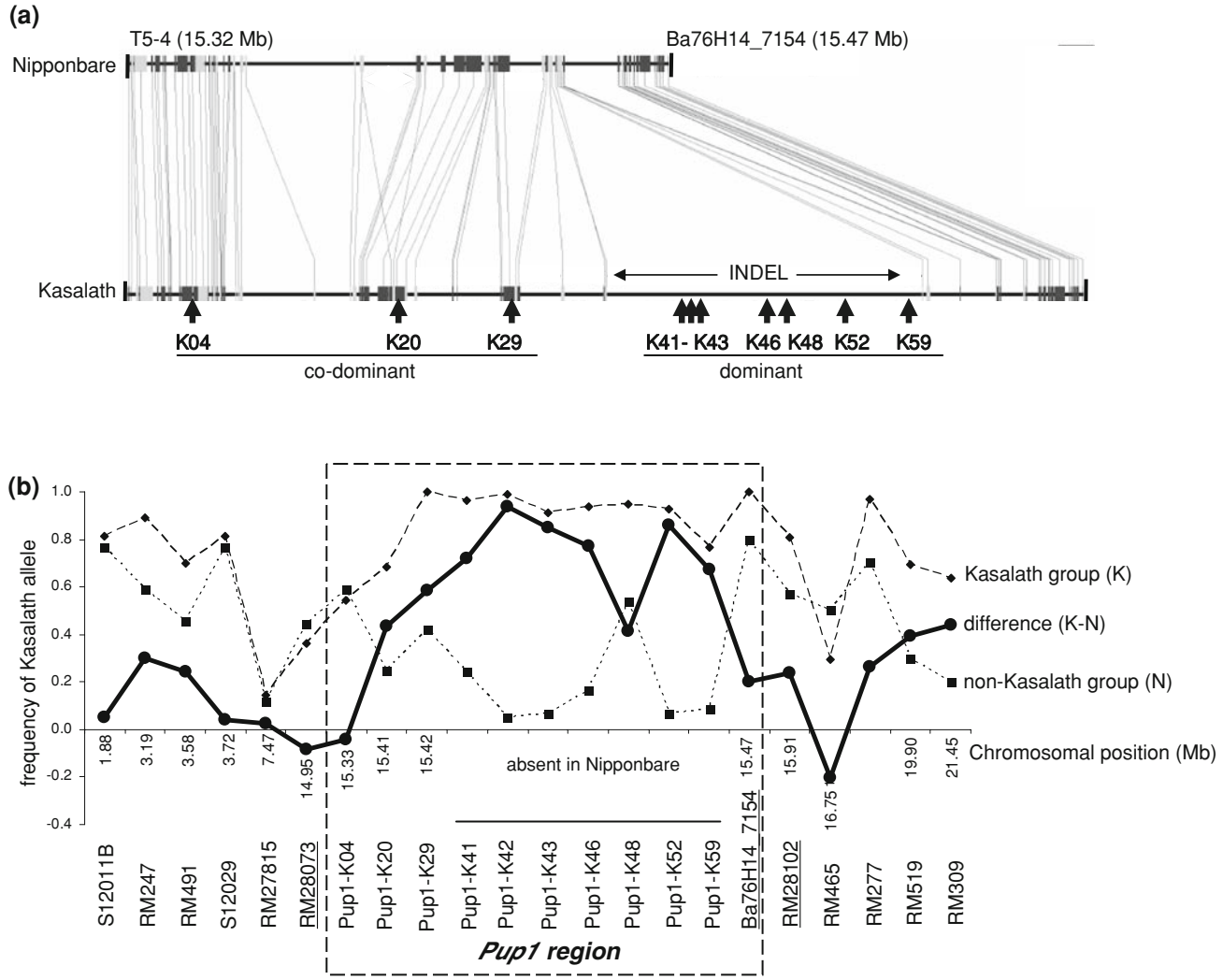

Fig. 1 Physical location of *Pup1* markers. The Kasalath *Pup1* genomic region (**a**, *bottom*) was aligned to the syntenic region in the TIGR5 Nipponbare reference genome (**a**, *top*) as defined by the *Pup1* fine-mapping markers T5-4 and Ba76H14_7154 (see Heuer et al. 2009 for details). Gene-specific markers were designed for nine *Pup1* putative genes indicated under the alignment. Markers K20 and K29 are co-dominant, whereas markers K41–K59 are located in a Kasalath-specific INDEL region and are therefore dominant for Kasalath. Additional markers located on Chr. 12 but outside of the *Pup1* region that were included in this study are shown in (**b**). The physical position of the markers is indicated in mega base pairs (Mb)

both traditional and modern varieties developed for unfavorable conditions, suggesting that upland rice breeders have unknowingly selected for *Pup1*. In *aus*-type varieties, *Pup1* is equally present in 80–90% of the upland and lowland/irrigated varieties. An exception within the *aus* group is the variety N22 with only two Kasalath alleles (Supplementary Table S1).

Pup1 haplotypes and field performance

To establish if the obtained *Pup1* genotypic data are correlated with P-uptake efficiency, we genotyped 19 accessions that were available at IRRI out of a total of 30 accessions that were included in the initial *Pup1* screening in Japan (Wissuwa and Ae 2001a). This screening was conducted in a P-deficient field (plant available P: 5 mg P kg^{-1} soil; Bray2) under rainfed conditions using a set of traditional and modern varieties of different geographical origin and genetic background. With respect to P-uptake, the authors provided data on P uptake under low- and high-P conditions, as well as on relative P uptake (Wissuwa and Ae 2001a; Table 2; Supplementary Fig. S3). We have reassessed this data set to determine the *Pup1* genotype × P-uptake efficiency. In agreement with the data from our germplasm survey, *Pup1* was found to be absent from all lowland/irrigated *indica* and *japonica* varieties that were included in the analysis (N-group). In contrast, all upland varieties, with the exception of Gaisen Ibaraki 2, possessed Kasalath alleles at all targeted loci (K-group). The latter group consisted solely of *aus*- and *japonica*-type

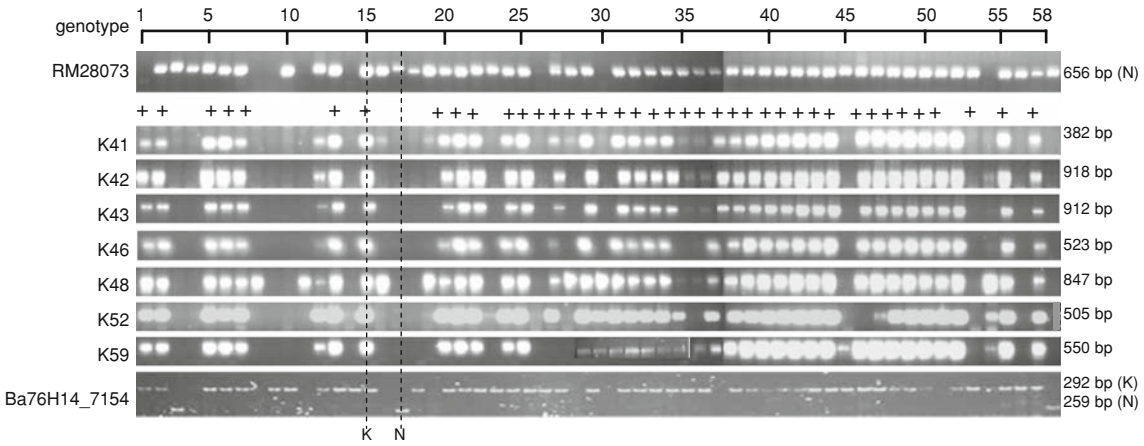

Fig. 2 *Pup1* haplotype of 58 rice genotypes. The *Pup1* haplotype was determined by genomic PCR using seven *Pup1* gene-based markers (K41–K59) and two closely flanking markers (RM28073, Ba76H14_7154). A representative subset of 58 rice genotypes is shown. Names are given in supplementary Table S1. All markers used for this study are dominant and amplify only Kasalath (K) alleles. The absence of PCR products indicates Nipponbare (N) or non-Kasalath alleles. Drought-tolerant breeding lines are indicated by (+). Size of DNA fragments is indicated in base pairs (bp). *W* water control

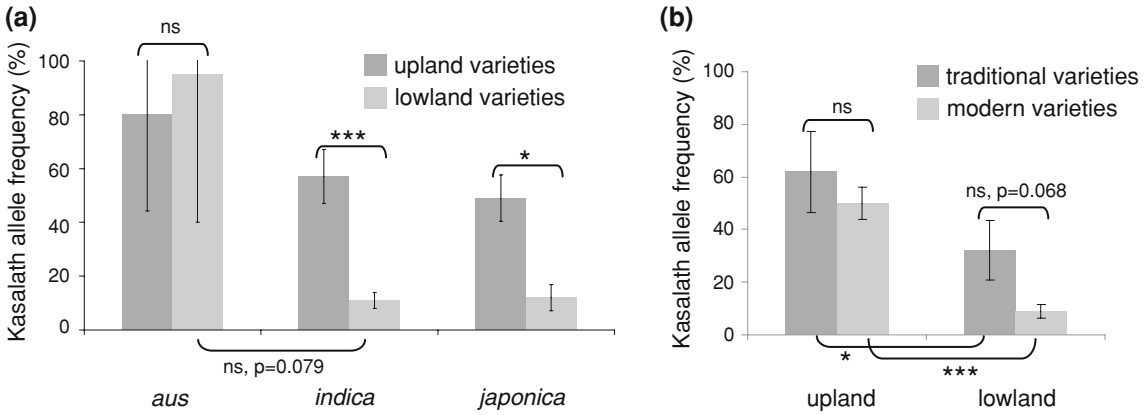

Fig. 3 *Pup1* haplotype survey. Rice accessions genotyped with the *Pup1* markers were grouped according to their preferred cropping systems and varietal group (*indica*, *japonica*, *aus*) (**a**). In (**b**) the same genotypes were grouped according to their classification as modern or traditional varieties. *Error bars* indicate standard error of the mean

traditional varieties (except Oryzica Sabana 6). No *indica* variety with *Pup1* was represented in this analysis but some should be included for comparison in future experiments. With respect to P-uptake efficiency, genotypes that possess *Pup1* alleles for the analyzed genes (K-group) accumulated significantly more P plant^{-1} under both, high-P ($p < 0.001$) and low-P ($p < 0.01$) conditions (Supplementary Fig. S3). Varieties of the K-group showed an average P uptake of 45.9 mg plant^{-1} which was significantly ($p < 0.001$) higher than in the N-group (average of 24.1 mg plant^{-1}). Likewise, differences in P uptake under low-P conditions were significantly ($p < 0.01$) higher in the K-group (7.3 mg P plant^{-1}) compared to the N-group (3.2 mg P plant^{-1}) (Table 2; Supplementary Fig. S3). Relative P-uptake values were similar in both groups (K-group: 15.9 mg plant^{-1}, N-group: 13 mg plant^{-1}). It should be noted that modern varieties with high-yield potential (e.g., Ashihikari, IR72) took up less P than traditional varieties even under high-P conditions (Table 2; Fig. 4). This might indicate that limited conclusions can be drawn from screening lowland/irrigated varieties under upland conditions since they generally show low adaptation to these environments and therefore might have suffered from stresses others than P deficiency, e.g., water limitation. This is again illustrated in Fig. 4, which shows that all analyzed lowland/irrigated varieties showed low P uptake under high-P conditions. The high relative P uptake (P uptake under low-P/P uptake under high-P conditions) observed in some of these varieties (e.g., IR36, IR72) therefore does not reflect efficient P uptake (see Table 2 for details). In contrast, the analyzed upland-adapted varieties consistently show high P-uptake values under high-P conditions and the majority of this group also reached high relative P-uptake values. These phenotypic data are well in

Table 2 *Pup1* genotype and phenotypic data on P-uptake of 19 rice genotypes

		N-group										K-group									
		Akihikari	Lemont	Sri Kuning	Nipponbare	Arkansas Fortuna	IR 72	IR 36	IR 66	Singgora	YS 27	Gaisen Ibaraki 2	Pratao Precoce	Oryzica Sabana 6	NIL C443	Kasalath[b]	Vary Lava 701	IAC 47	IAC 25	Dular	Average LL varieties	Average UL varieties
P uptake mg plant^{-1}[a]	Low-P conditions	0.7	0.6	1.1	2.2	2.7	3.9	5.4	5.2	4.9	4.7	3.6	2.3	1.5	6.1	9.1	9.2	9.1	9.6	12.9	3.2**	7.3
	High-P conditions	10.0	18.7	20.9	28.0	32.5	17.3	20.1	27.5	28.5	36.4	32.7	36.9	49.2	40.6	40.1	58.3	66.7	37.0	46.1	24.1***	45.9
	Relative	7.0	3.2	5.3	7.9	8.3	22.6	26.9	18.8	17.2	12.9	11.0	6.2	3.0	15.0	22.7	15.8	13.6	26.0	28.0	13.0 ^{ns}	15.9
Genetic and environmental background	Cropping system	L	L	L	L	L	L	L	L	L	L	U	U	U	L/U	L/U	U	U	U	L/U		
	Varietal group	J	J	J	J	I	I	I	I	I	J	J	J	J	J	A	J	J	J	A		
	History	MV	MV	TV	MV	MV	MV	MV	MV	TV	TV	TV	TV	MV	MV	TV	TV	TV	TV	TV		
Name of *Pup1* putative gene	Marker name																					
OsPupK41-1	*Pup1*-K41	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K	K	K		
OsPupK42-1	*Pup1*-K42	N	N	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K		
OsPupK43-1	*Pup1*-K43	N	N	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K		
OsPupK46-1	*Pup1*-K46	N	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K	K		
OsPupK48-1	*Pup1*-K48	N	K	K	N	N	K	K	N	N	N	K	K	K	K	K	K	K	K	K		
OsPupK52-1	*Pup1*-K52	N	N	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K		
OsPupK59-1	*Pup1*-K59	N	N	N	N	N	N	N	N	N	N	N	K	K	K	K	K	K	K	K		

L lowland, *U* upland, *J japonica*, *I indica*, *A aus*, *MV* modern variety, *TV* traditional variety, *N* non-Kasalath allele, *K* Kasalath allele, *ns* not significant

**Significant at $p < 0.01$, ***significant at $p < 0.001$. See also Supplementary Fig. S3

[a] Data from Wissuwa and Ae (2001a)

[b] *Pup1* introgression line

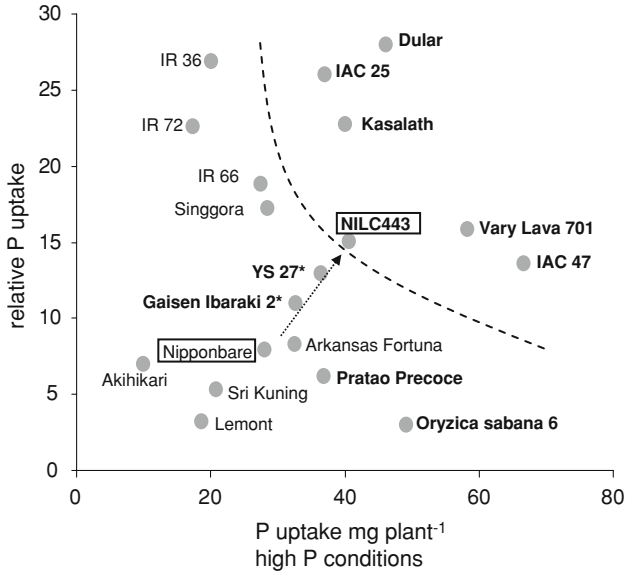

Fig. 4 P uptake in rice varieties with and without the tolerant *Pup1* haplotype. P uptake under P-deficient and P-fertilized conditions as reported by Wissuwa and Ae (2001a) was compared between rice varieties with the *Pup1* locus (*bold characters*) and without the *Pup1* locus. Relative P uptake indicates % P uptake under P-deficient conditions in relation to P uptake under P-fertilized conditions (see Table 2 for details). The near-isogenic *Pup1* introgression line NILC443 derived from a Nipponbare × Kasalath (tolerant, *Pup1* donor) population showed higher P uptake under high-P conditions and a higher relative-P uptake in comparison to the intolerant irrigated parent Nipponbare (indicated by *arrow*)

agreement with the *Pup1* genotypic data showing the presence of *Pup1* only in the latter group. The lowest P-uptake value in this group was obtained by Gaisen Ibaraki, a variety with partial *Pup1* (Table 2).

To examine whether *Pup1* has the potential to improve P uptake under upland conditions in an intolerant irrigated variety, we have included data from Nipponbare and the derived NIL-C443, which carries *Pup1* plus some additional Kasalath introgressions (Wissuwa et al. 2002). Based on this analysis, introgression of *Pup1* into the genetic background of a P-inefficient irrigated variety appears to have the potential to significantly increase both P uptake under P-fertilized upland conditions (P responsiveness) and relative P uptake (P-deficiency tolerance; Fig. 4).

Pup1 phenotyping in hydroponics and Philippine P-deficient soil

The above data provide first evidence that *Pup1* is a QTL with the potential to improve P uptake under adverse conditions and that it is beneficial in diverse genetic backgrounds. As a next step, it is important to demonstrate that *Pup1* is stable across different environments and to develop a robust phenotyping system. Since field experiments under tropical short-day conditions are prevented by the photosensitive Nipponbare background, we have evaluated the *Pup1* NILs in a hydroponics and a soil-based pot experiment. The hydroponics experiment was conducted to assess differences in the root system between different pairs of contrasting *Pup1* NILs in comparison to rice varieties adapted to upland or irrigated cropping systems. Plants were grown in hydroponics solution under P-deficient and control conditions (0 and 100 μM P). Since an increase in root length is considered a general response to P deficiency in rice (Shimizu et al. 2004; Li et al. 2009), we have used this parameter to assess genotypic differences. Indeed, large genotypic variations in the root system and an increase in root length in response to P deficiency were observed (Fig. 5a, b; Supplementary Table S2). Although some varieties responded with an increase in root length when grown under P-limiting conditions (e.g., Dular, Jalmagna, IAC 47, and IAC 25), others showed low or no response (e.g., Vary Lava 701, YS27, IR36, IR66, and IR72). In agreement with earlier observations (Wissuwa and Ae 2001b; Wissuwa 2005), this is independent of *Pup1* since NILs with and without *Pup1* showed a similar response (compare NIL6-4/6-3 and NIL14-4/14-6, Fig. 5a, b). Furthermore, the observed differences in root growth did not seem to have a significant effect on shoot growth since all genotypes, with the exception of YS 27, showed around a 50% reduction in shoot length (Fig. 5c; Supplementary Table S2). This experiment further revealed differences in the root system of the two pairs of *Pup1* NIL sister lines. Whereas NILs 6-3 and 6-4 are similar to Nipponbare, NILs 14-4 and 14-6 possessed a root system similar to Kasalath. This is likely due to an additional introgression on Chr. 8 present in NIL14-4 and NIL14-6 but absent in NIL6-4 and NIL6-3 (see below; Supplementary Fig. S2). A detailed comparative marker analysis is now ongoing to map the corresponding genomic regions.

Based on the hydroponics experiment, we have chosen the contrasting NILs 14-4 and 14-6 for further soil-based analyses. Using NILs with a large root system comparable to those found in upland adapted varieties ensures that phenotypic differences under P-limiting conditions are due to the effect of *Pup1* and not to a large root system per se. In the first soil-based experiment, plants were grown under well-watered but aerobic (non-flooded) conditions, accounting for the fact that *Pup1* might be an upland-associated QTL (see above), and data reported by Wissuwa and Ae (2001b) showing that the *Pup1* phenotype was undetectable in paddy fields. In agreement with this, we were unable to reproducibly observe the *Pup1* phenotype in flooded soil (data not shown). Plants were additionally exposed to long-day conditions (15.5–16 h light) for about 6 weeks to induce tillering. As shown in Fig. 6a, under

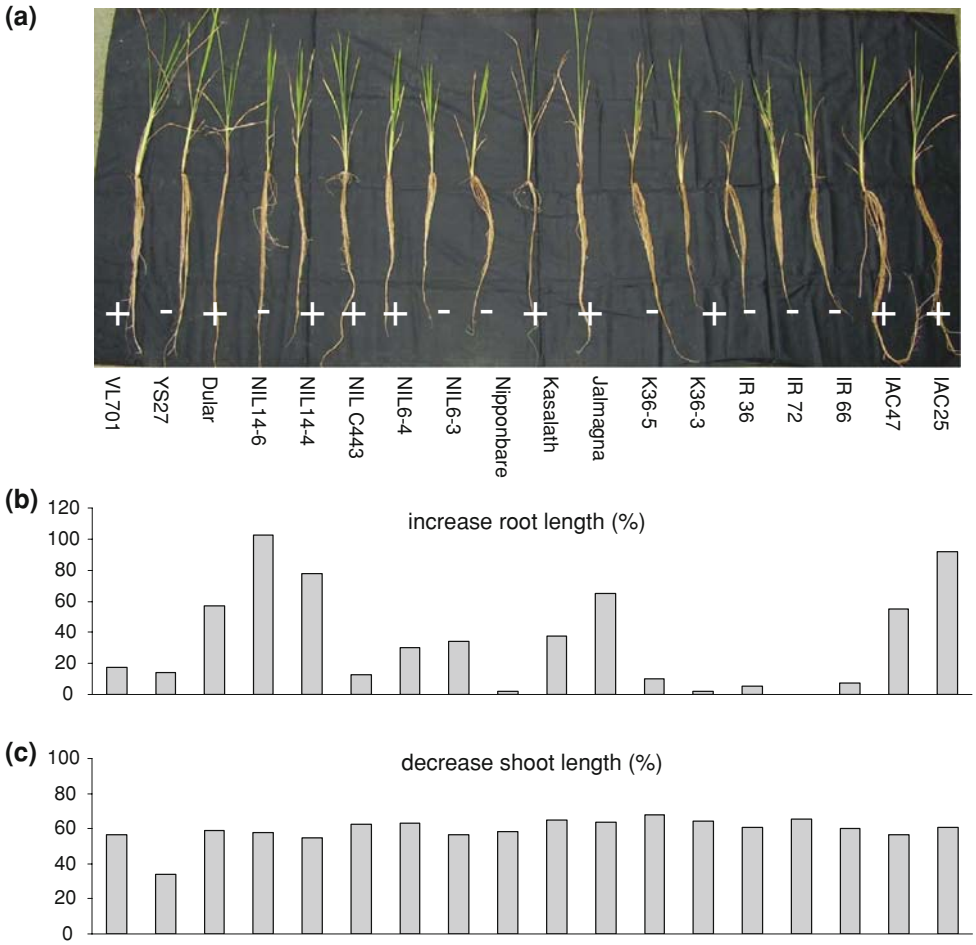

Fig. 5 Root elongation and reduction in shoot length under P deficiency in hydroponics culture solution. A set of contrasting *Pup1* near-isogenic lines with the *Pup1* locus (NILC443, NIL6-4, and NIL14-4) and sister lines without the *Pup1* locus (NIL6-3, NIL14-6) were grown in hydropnics solution with 0 and 100 μM P, respectively. A subset of the varieties phenotyped by Wissuwa and Ae (2001a) was additionally included in the experiment. The photo in (**a**) shows the plants after 52 days of growth in hydroponics without P. Presense (+) and absence (−) of the *Pup1* locus is indicated. The increase in root length (**b**) and reduction in shoot length (**c**) under 0 μM P conditions compared with 100 μM P conditions are shown. See also Supplementary Table S2

these conditions, no significant differences between the NILs with respect to tiller number, plant height, and root length were detected between ±P treatments at the two analyzed time points. In contrast a reduction in shoot and root-dry weight as well as a reduced grain yield were observed under P deficiency in both NILs (Fig. 6b–d). Though the obtained data show relatively high standard deviations, the adverse effect of P deficiency was less pronounced at maturity in the *Pup1* NIL14-4 which showed a significantly ($p < 0.05$) lower average reduction in shoot (32.5%) and root (21.3%) dry weight compared to NIL14-6 (reduction in shoot dry weight by 47.9%, root dry weight by 52.5%). The data on grain number and total grain weight per plant were less variable and showed significant ($p < 0.01$) differences between the NILs (Fig. 6c, d). Under P-deficient growth conditions, NIL14-6 showed a reduction in grain number and grain weight plant^{-1} by 36 and 60.3%, respectively, whereas the *Pup1* NIL14-4 showed a reduction in grain number by only 6% and a reduction in total grain weight plant^{-1} by only 33.5%. The higher reduction in grain weight than in grain number in NIL14-4 can be explained by partial grain filling. Interestingly, NIL14-4 outperformed the intolerant sister line NIL14-6 even under +P conditions. This was also observed in an independent pot experiment, as well as in a screening of rice accessions with contrasting *Pup1* haplotypes under lowland +P field conditions (data not shown). Because of the photosensitive Nipponbare background, field screenings with the *Pup1* NILs could not be conducted in the Philippines.

The beneficial effect of *Pup1* has been independently confirmed using P-deficient Japanese soil and three *Pup1* NILs (Heuer et al. 2009) (Fig. 6e, f). Plants were grown under well-watered aerobic conditions with and without P fertilizer application. The data show that the presence of the *Pup1* locus significantly ($p < 0.05$) increased grain weight and P content plant^{-1} under P-deficiency (Fig. 6e, f). Under P-fertilized conditions, no significant differences were detected.

In order to validate that the observed differences between the contrasting NILs are due to the presence of *Pup1* and not due to any additional Kasalath introgression, the background genotype was determined using the GoldenGate SNP-marker platform (Zhao et al., Cornell University, personal communication). This analysis revealed small regions of Kasalath introgressions that differ between the analyzed NILs and confirmed that

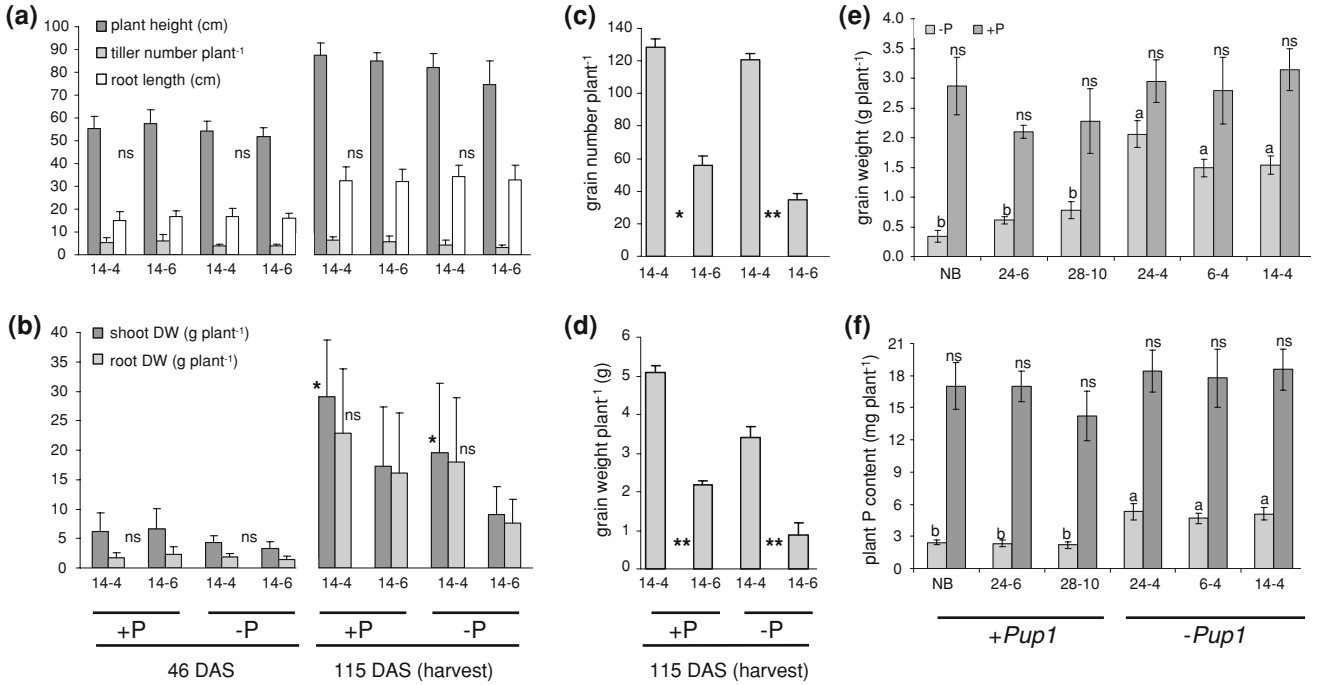

Fig. 6 *Pup1* phenotype in P-deficient soil. The contrasting *Pup1* sister lines NIL14-4 (+*Pup1*) and NIL14-6 (−*Pup1*) were grown in P-deficient Philippine soil (−P) without and with P-fertilizer (+P) application under well-watered but non-flooded (aerobic) conditions. Plant height, tiller number, and root length (**a**) as well as shoot and root dry weight plant^{-1} (**b**) were measured at 46 and 115 days (harvest) when grain number plant^{-1} (**c**) and grain weight plant^{-1} (**d**) were determined. In an independent experiment Nipponbare (NB) and NILs with contrasting *Pup1* haplotypes (+*Pup1*: NIL6-4, NIL14-4, NIL24-4; −*Pup1*: NIL14-6, NIL24-6; Heuer et al 2009) were grown in P-deficient Japanese soil with (+P) and without (−P) P-fertilizer. Grain weight (**e**) and plant P content (**f**) was determined at maturity. *Error bars* indicate standard deviations of means; significance levels: *ns* non significant, *significant at $p < 0.05$, ** significant at $p < 0.01$. Within P treatments, different letters signify significant differences according to Tukey's HSD test at $p < 0.05$

phenotypic differences are caused by the presence or absence of the *Pup1* region (Supplementary Fig. S2).

In summary, the obtained data demonstrated that the *Pup1* phenotype can be observed in at least two different environments and soil types, and that it confers tolerance of P deficiency under the applied screening conditions. Further optimization of the phenotyping protocol is ongoing.

Discussion

Pup1 molecular markers

Based on the available *Pup1* genomic sequence and a preliminary gene prediction (Heuer et al. 2009), we have designed gene-based markers that target two structurally different regions in the *Pup1* locus. The co-dominant markers target genes located in regions partially conserved in the Nipponbare reference genomes, whereas the dominant markers target genes in an INDEL region that is absent in Nipponbare. The germplasm survey conducted with these markers showed that most of the dominant markers are highly diagnostic for *Pup1* across a large number of genotypes. Some of the analyzed accessions seemingly possessed a truncated or rearranged *Pup1* locus since only a few Kasalath alleles of the genes present in the INDEL region were detected. Particularly, marker K48 showed little diagnostic value although the adjacent markers K46 and K52 were highly diagnostic for *Pup1*. This might be explained by the fact that K48 is located in direct proximity to a region within the Kasalath *Pup1* locus that could not be assembled due to a high frequency of repetitive sequences (Heuer et al. 2009). It is therefore possible that marker K48 is unspecific in some accessions. Likewise, the two co-dominant markers that were tested in this study were less diagnostic across genotypes, suggesting that the targeted polymorphisms in the two genes are not functionally responsible for tolerance. Since the actual *Pup1* major tolerance gene(s) has not yet been identified, allelic sequencing of partially conserved *Pup1* genes in representative tolerant and intolerant rice accessions is needed to determine functional polymorphism. This work is currently ongoing at IRRI. Based on the above data, the dominant markers, with the exception of marker K48, are suitable for the identification of recipient parents and detection of *Pup1* introgressions in breeding programs using diverse parental lines. The analysis of the *Pup1* flanking marker that was used for fine mapping *Pup1* in a

Kasalath × Nipponbare population (Heuer et al. 2009) revealed that this marker is highly monomorphic within the analyzed germplasm. To delineate the *Pup1* introgression in other breeding lines, it is therefore necessary to identify variety-specific flanking markers.

The above data exemplify the complexity of marker development and especially emphasize the importance of sequencing a given QTL in the respective donor parent. The INDEL region that harbors the most diagnostic markers as well as several putative candidate genes (Heuer et al. 2009) is absent from the Nipponbare reference genome. The same is true for the major submergence-tolerance gene, *SUB1A* (Xu et al. 2006). These findings have some implications for future high-throughput genotyping platforms since genomic regions that are absent from reference genomes are not eligible for, e.g., SNP or INDEL marker development, which rely on differences between genes present in at least two genomes. Though only sequencing of the genomic region harboring a given QTL will ensure that novel genes do not remain unidentified, ongoing next-generation sequencing of additional reference genomes and rice varieties will help to overcome this problem in the near future (e.g., OMap, http://www.omap.org/). Additional sequence information will also facilitate more rapid identification of functional polymorphism between alleles in tolerant and intolerant genotypes.

Pup1 germplasm screening

The germplasm survey conducted with the developed markers revealed that *Pup1* is conserved in most *aus* varieties, as well as in *japonica* and *indica* upland varieties, whereas it is absent from the majority of *indica* and *japonica* varieties developed for favorable irrigated conditions. The fact that the presence or absence of *Pup1* is not varietal-group specific but is related to different rice agro-systems excludes the possibility that *Pup1* was eliminated from modern varieties during ancient or more recent domestication bottlenecks (Ma and Bennetzen 2004). Rather, it appears that breeders have actively, though unknowingly, selected for *Pup1* for unfavorable environments. This selection seems to be still ongoing since *Pup1* is present in modern upland varieties whereas it is absent from modern irrigated varieties. Alternatively, it is possible that *Pup1* is linked to some undesirable traits and that breeders have therefore selected against *Pup1* for high-yielding environments. Linkage drag is a common problem encountered by breeders but it can now be overcome by using closely linked markers that facilitate breakage of linkage (e.g., Liu et al. 2009). In order to address this question, it will be necessary to compare the performance of *Pup1* NILs to that of the respective recipient parent under favorable irrigated conditions. Results from trials conducted in Japan suggest that Nipponbare-based *Pup1* NILs do not have a linkage drag. However, since our target is to use *Pup1* for the improvement of tropical rice, this question will be considered once *Pup1* NILs become available in the IR64 background (see below).

One of the most promising findings of this study is that *Pup1* is present in more than 80% of the analyzed drought-tolerant accessions. Though more detailed analyses are needed to exclude the possibility that drought tolerance might be conferred by additional QTLs present in these accessions, a positive effect of *Pup1* on drought tolerance is likely since it was mapped under upland field conditions (Wissuwa and Ae 2001a; Wissuwa et al. 2002). In addition, it was reported that the *Pup1* phenotype is undetectable in hydroponics culture solution and not expressed under irrigated (paddy) field conditions (Wissuwa and Ae 2001b). These data are in agreement with our hydroponics experiment as well as the observation that phenotypic differences between contrasting *Pup1* NILs are best expressed under aerobic conditions (see below).

As is the case for *Pup1*, other large-effect QTLs were mapped in traditional *aus*-type varieties. The *aus* group is phylogenically close to *indica* rice (Garris et al. 2005), but historically developed in a region with high occurrence of poor soil (Londo et al. 2006; Haefele and Hijmans 2007). It is therefore not surprising that favorable alleles for stress tolerance have evolved and are still present in this group. Prominent examples are *Sub1* which was identified in FR13A, and *Saltol*, which was mapped in Pokkali (Walia et al. 2005; Ren et al. 2005). Recent SNP genotyping data revealed that the Pokkali *Saltol* region likely represents an introgression of an *aus* variety present in the Pokkali pedigree (K. McNally, personal communication). Likewise, tolerance to high temperature at flowering stage has been reported in N22, a variety from India that is commonly considered an *aus* variety (Reddy et al. 2009). However, whether N22 is correctly classified has recently been questioned by SSR genotyping data that failed to group N22 with other *aus* varieties (Garris et al. 2005). Likewise, our *Pup1* genotypic data showed that N22, in contrast to other *aus* varieties, possessed only a few Kasalath alleles at *Pup1*. More detailed analyses of a larger number of *aus* varieties, and especially comparative analysis of the eight different N22 accessions that are registered in the IRRI gene-bank collection are needed to draw final conclusions.

The disadvantage of *aus* varieties and tolerant landraces is their overall poor agronomic performance, which makes them less suitable for breeding programs. Without marker selection, the risk is high that favorable alleles still present in F1 generations are lost during repeated backcrossing necessary to restore a desirable plant type. Molecular markers have meanwhile been successfully applied in the

development of *Sub1* and *Saltol* introgression lines (Septiningsih et al. 2009; IRRI unpublished data), and the markers reported here are now being used for *Pup1* introgression into irrigated (IR64, IR74; Heuer et al., unpublished) and Indonesian upland varieties (J. Prasetiyono and M. Bustaman, unpublished data).

Phenotyping of Pup1 near isogenic lines

The validation of QTLs in different environments and genetic backgrounds is one of the most important steps toward their large-scale application in breeding programs. Only QTLs that express their positive effect independent of the environment will be of interest to breeders. The diversity of rice cultivars and specific local preferences on, e.g., grain quality furthermore requires breeders to develop locally adapted varieties, and it is therefore essential that QTLs are beneficial across different rice varieties and environments. The phenotypic differences between contrasting *Pup1* NILs reported here confirmed data obtained in Japan in an extended set of NILs (Wissuwa and Ae 2001a; Wissuwa 2005) and for the first time showed the beneficial effect of *Pup1* in a different environment and soil type. In contrast to earlier data obtained in field and pot experiments in Japan, where differences in plant height and tiller number were observed between contrasting NILs during the vegetative growth phase (M. Wissuwa, personal communication; Wissuwa and Ae 2001b), we observed differences between the NILs only at maturity. At this stage, *Pup1* NIL14-4 significantly outperformed the intolerant sister line NIL14-6 by maintaining a higher total grain number and grain weight plant^{-1} under P deficiency. Overall, our screening conditions are less stringent than field experiments reported from Japan where even tolerant lines (NILC443 and Kasalath) showed yield reductions of about 80% under −P conditions (Wissuwa and Ae 2001a) compared with a reduction in grain weight plant^{-1} of about 30% (NIL14-4) and 60% (NIL14-6) reported here. This might be due to the slightly higher P content of the Philippine soil ($\sim$6 mg P kg^{-1} soil) compared with the soil used in Japan ($\sim$5 mg P kg^{-1} soil) and a more regular water supply.

Under the applied screening conditions, a yield advantage of NIL14-4 was also observed under +P control conditions using Philippine soil. This was also seen in an independent pot experiment, as well as in a survey of genotypes with different *Pup1* haplotypes conducted under +P field conditions (data not shown). This finding is further in agreement with the initial phenotypic data reported from Japan, which showed that varieties with the *Pup1* locus showed higher P uptake under both low-P and high-P conditions (Table 2; Wissuwa and Ae 2001a). A beneficial effect of *Pup1* under P-fertilized and non-fertilized conditions would be ideal since it ensures maximum return from any amount of P fertilizer applied.

A possible explanation for the observed benefit of *Pup1* under +P conditions is that P availability may become critically low even under fertilized conditions during the intermittent periods of more or less severe water stress typically encountered under aerobic (rainfed) growth conditions. This is because P diffusion in dry soil is severely impaired and P might therefore not be plant-available (e.g., Rodriguez and Goudriaan 1995). Under these circumstances, *Pup1* would be beneficial and would indirectly improve drought tolerance. Recently, a major QTL (qtl12.1) for yield under drought has been mapped in a Vandana × Way Rarem population (Bernier et al. 2007, 2009). This QTL is located on Chr. 12 between 14.1 and 17.4 Mb according to the Nipponbare reference genome and therefore overlaps with *Pup1*, which is located at 15.4 Mb. Work is now in progress to address the possibility that these two QTLs might indeed be identical and that *Pup1* may be a crucial component of the genetic makeup of rice genotypes with tolerance of the multiple stresses encountered in drought-prone environments.

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