RESEARCH ARTICLE

DNA sequence polymorphism of *Pup1* linked *K20-1* STS region can be effectively used in molecular breeding of rice for phosphorus deficiency tolerance

Y.C. Aluwihare^{1,2}, M.D.M. Chamikara¹, D.R.R.P. Dissanayake¹, M.D.M.I.M. Dissanayake¹, N.N.H. Karannagoda¹, A.G.M.L.K. Dayananda¹, D.N. Sirisena³, W.L.G. Samarasinghe³, R.G.S.C. Rajapakse^{1,2} and S.D.S.S. Sooriyapathirana^{1,2*}

¹ Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Peradeniya.

² Postgraduate Institute of Science, University of Peradeniya, Peradeniya.

³ Rice Research and Development Institute, Bathalagoda, Ibbagamuwa.

Revised: 16 June 2017; Accepted: 20 July 2017

Abstract: *Pup1* is the major quantitative trait locus (QTL) for phosphorus deficiency (PD) tolerance in rice. The molecular markers linked to *Pup1* are available for numerous rice genotypes. However, novel haplotypes of *Pup1* could be detected if specific landraces are subjected to genetic analyses. A set of 30 local rice genotypes were assessed for PD tolerance and in the present study, they were characterised by sequencing *K20-1*, a co-dominant locus within the *Pup1* locus. The DNA polymorphism was also checked for the association with PD tolerance. A total of 10 SNPs and one INDEL were discovered among the 32 genotypes including *Kasalath* and *Nipponbare* as PD tolerant and sensitive reference rice varieties, respectively. Four unique SNPs were discovered in the studied germplasm that are not present in either *Kasalath* or *Nipponbare*. The SNP located at the 209th position was very important as it is unique to highly PD tolerant landraces *Murungkayan* and *Suduheenati*, and varieties H-4, H-7 and H-10. These five genotypes lack the INDEL region present in *Kasalath* highlighting the presence of novel *Pup1* haplotypes in country specific rice varieties/landraces, and they can be used as parental genotypes to introduce PD tolerance into new varieties in rice breeding programmes in Sri Lanka.

Keywords: *K20-1*, marker assisted breeding, *OsPupK20-2*, phosphorus deficiency tolerance, *Pup1*, rice landraces.

INTRODUCTION

Phosphorus (P) is one of the key limiting nutrients that can significantly affect profitable rice farming (Fageria *et al.*, 1982; Hedley *et al.*, 1994; Kirk *et al.*, 1998). The application of artificial P fertiliser is very expensive, which could also contribute to environmental pollution and health hazards (Bennett *et al.*, 2001; Cordell *et al.*, 2009; Chandrajith *et al.*, 2010; Jiao *et al.*, 2012). The development of improved rice varieties that can perform well under low P regimes using marker assisted breeding (MAB) is identified as the most promising and sustainable solution to tackle these problems (Wissuwa, 2003). Although MAB programmes for P deficiency (PD) tolerance are not yet routinely employed in rice breeding, many physiological and genomic studies have been carried out. PD tolerant and sensitive rice landraces and improved varieties have been identified in countries such as China (Li *et al.*, 2009), the Philippines (Heuer *et al.*, 2009), Indonesia (Prasetyono *et al.*, 2010), India (Tyagi *et al.*, 2012) and Sri Lanka (Aluwihare *et al.*, 2016). The genomic basis of PD tolerance has been subjected to intense studies and a quantitative trait locus

* Corresponding author (sunethssuop@gmail.com; <https://orcid.org/0000-0002-5592-1742>)

This article is published under the Creative Commons CC-BY-ND License (<http://creativecommons.org/licenses/by-nd/4.0/>). This license permits use, distribution and reproduction, commercial and non-commercial, provided that the original work is properly cited and is not changed anyway.

(QTL), *Pup1*, with a major effect, has been identified on rice chromosome 12. Another QTL with a minor effect was also identified, which is located on chromosome 6 (Wissuwa *et al.*, 1998). These QTLs have been initially detected using a drought and PD tolerant rice landrace, *Kasalath* and later validated using a PD sensitive landrace *Nipponbare* derived near-isogenic lines (NILs) (Wissuwa & Ae, 2001; Wissuwa *et al.*, 2002). It was reported that the increasing alleles/haplotypes of *Pup1* could increase the yield by two- to four-fold (Wissuwa, 2003), which is a significant reward in rice farming if put into the practice through MAB. The mechanism of PD tolerance conferred by *Pup1* has been further studied (Wissuwa, 2005; Yi *et al.*, 2005; Gamuyao *et al.*, 2012) and the differences of the root surfaces were characterised among the tolerant and sensitive rice genotypes (Wissuwa, 2003; Wissuwa *et al.*, 2005). It was found that *Pup1* is co-segregating with the external efficiency of P uptake (Wissuwa, 2005). A larger effect of *Pup1* conferred tolerance is mainly found to act on roots and the candidate genes within the *Pup1* region were also identified (Heuer *et al.*, 2009; Chin *et al.*, 2011). In addition to PD tolerance, *Pup1* was also reported to confer drought tolerance in rice (Ismail *et al.*, 2007; Chin *et al.*, 2011). Many attempts have been made to develop molecular markers linked to *Pup1* region that can be used in MAB (Heuer *et al.*, 2009; Chin *et al.*, 2010; Chin *et al.*, 2011). Pariasca-Tanaka *et al.* (2014) have developed an allele specific marker to transfer *Pstol1* gene (coding for a *Pup1* specific protein kinase present only in PD tolerant genotypes) from the donor parent *Kasalath* to PD sensitive African rice varieties. A marker haplotype analysis for *Pup1* region has been conducted using a set of rice germplasm available in India (Tyagi *et al.*, 2012). However, the most comprehensive genomic analysis of *Pup1* was conducted by Chin *et al.* (2011) and they developed molecular markers that could be used to detect allelic haplotypes and associated sequence polymorphisms. Furthermore, comprehensive bioinformatic and functional genomic studies on the key genes identified within the *Pup1* locus were also conducted (Yi *et al.*, 2005; Chin *et al.*, 2011; Gamuyao *et al.*, 2012). However, many promising landraces and improved varieties were not included as the sources for mapping in those studies.

Recently a set of PD screening results of a selected panel of germplasm including landraces and improved rice varieties in Sri Lanka was published (Aluwihare *et al.*, 2016). The present study was conducted to identify whether the genotypes studied by Aluwihare *et al.* (2016) contain similar or different haplotypes to that of PD tolerant *Kasalath* or sensitive *Nipponbare* landraces using the co-dominant, easily amplifiable and polymorphic *K20-1* locus of the *Pup1* genomic region

reported in Chin *et al.* (2011). The associations between DNA sequence polymorphism within the *K20-1* region and PD tolerance related traits were also examined to establish a basis for MAB of rice.

METHODOLOGY

Rice genotypes and assessment of PD tolerance

A total of 30 rice genotypes (Table 1) including landraces and improved varieties obtained from the Rice Research and Development Institute (RRDI), Bathalagoda, Sri Lanka were used in the present study. The plants were grown under P_0 [using the ultisol soil (1 mg of P in a kg of soil) at RRDI, which has not been fertilised for the last 40 years (Kumaragamage & Indraratne, 2011)] and P_{30} [recommended P fertiliser application rate (30 mg of P_2O_5 was added to 1 kg of ultisol soil)] conditions under two main annual cropping seasons in Sri Lanka: *Yala* and *Maha* seasons (DOA, 2006a). The experiments were conducted under greenhouse and field conditions in 2012.

Growth parameters

Plant height (PIH) was measured from the base of the plant to its top using a meter ruler. The number of tillers (NT) was counted. PIH and NT data were recorded at the flowering stage of each genotype. Shoot dry weight (SDW) was obtained from the shoots of the plants harvested at the same stage. The shoots were briefly rinsed with distilled water and oven dried at 60 °C.

Trait related to P deficiency tolerance

Shoot P concentration (SPC)

Finely ground shoot material was liquefied in a solution containing 1 HNO_3 : 3 $HClO_4$: 1 H_2SO_4 and shoot P concentration (SPC) (mg of P/g of shoot dry matter) was determined according to the phosphovanadate procedure (Hanson, 1950).

Shoot P uptake (SPU)

Shoot P uptake (SPU) was calculated according to the formula explained in Fageria *et al.* (1988) and Gunes *et al.* (2006). The SPU was calculated collectively for whole four plants grown in the pot.

P utilisation efficiency (PUE)

P utilisation efficiency (PUE) was calculated as the biomass produced per unit P accumulated in the shoot tissue.

Table 1: Rice genotypes characterised for the DNA sequence polymorphism in *K20-1* locus

Type of cultivar	Name of the genotype ^a	PD tolerance score ^b
Landrace	<i>Suwandel</i>	2
	<i>Murungakayan</i>	3
	<i>Sudubalawee</i>	3
	<i>Suduheenati</i>	2
	<i>Hondarawala</i>	2
	<i>Marss</i> ^c	3
	<i>Kokuwellai</i>	3
	<i>Kaluheenati</i>	3
	<i>Pokkali</i>	2
	<i>Rathuheenati</i>	2
	<i>Rathel</i>	3
	<i>Sudurusamba</i>	2
Old improved varieties ^c	H-10	3
	H-4	3
	H-7	3
Newly improved varieties ^d	At 353	2
	At 306	1
	At 362	3
	At 354	1
	Bg 403	3
	Bg 250	2
	Bg 358	2
	Bg 357	1
	Bg 300	1
	Bg 450	2
	Bg 94-1	3
	Bg 352	2
	Bg 379-2	2
	Bw 364	2
	Ld 356	2

^a Standard rice variety names as given in the Database of Rice Varieties, Department of Agriculture, Sri Lanka (2006b)

^b PD tolerance scores are indicated as described in Aluwihare *et al.* (2016). 3: Tolerant; 2: moderately tolerant; 1: sensitive

^c Rice varieties developed before 1970

^d Rice varieties developed after 1970

^e *Marss* is also known as *Mas*

The performance of each genotype to P starvation was evaluated and analysed using multiple regression procedures to assign overall PD tolerance scores to each genotype (Aluwihare *et al.*, 2016) (Table 1). The same dataset was used in the current study for association analyses.

PCR and DNA sequencing

Leaf samples were collected from each rice genotype and the DNA was extracted using Dneasy® plant mini kit (Qiagen, Solna, Sweden). The DNA was PCR amplified using *K20-1* locus specific forward

(5'TCAGGTGATGGGAATCATTG3') and reverse (5'TGTTCCAACCAAACAACCTG3') primers (Chin *et al.*, 2011) using a thermal cycler (Takara, Japan). The PCR cycle consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 90 s, and 72 °C for 2 min, and a final extension step of 72 °C for 10 min. PCR products were visualised using 1 % agarose gel electrophoresis and purified using Wizard SV gel® and PCR clean-up system (Promega Corporation, Madison, Wisconsin, USA). The purified PCR products were cycle sequenced using a Sanger Sequencing based ABI 3500 Series Genetic Analyser (Applied Biosystems®).

Data analysis

Sequence alignment

As reference DNA sequences of *K20-1*, the GenBank entry for *Kasalath* (AB458444.1) (PD tolerant genotype) and *Nipponbare* (AP014968.1) (PD sensitive genotype) (www.ncbi.nlm.nih.gov/genbank/) were used. The *K20-1* DNA sequences of the 32 rice genotypes (including two reference sequences) were aligned using Clustal Omega software (<http://www.ebi.ac.uk>). The SNP and INDEL positions were identified and their allelic states were recorded for each genotype. The part of the exon 2 in *OsPupK20-2* gene falling within the *K20-1* region was translated to amino acid sequence and subjected to multiple sequence analysis using Clustal Omega.

Assessment and visualisation of DNA polymorphism

The polymorphic SNPs and INDEL present in 32 rice genotypes were subjected to cluster analysis and dendrogram construction using the statistical package Minitab 16 (Minitab Inc., USA). Clustering details of the genotypes were compared with the PD tolerance score data of the same genotypes published in Aluwihare *et al.* (2016).

Visualisation of identical by descent haplotypes and PD tolerance scores

The *K20-1* haplotypes of the genotypes were visualised using Pedimap software (Voorrips, 2007) to show the inheritance of haplotypes, identical by descent (IBD) probabilities and PD tolerance scores.

Analysis of the association between sequence polymorphism and PD tolerance traits

The PD tolerance screening data, PIH, NT, SDW, SPC, SPU and PUE, of the 30 rice genotypes were subjected to SNP/INDEL association analyses employing the GLM procedure in Tassel 5 software (Bradbury *et al.*, 2007). The mean differences between the phenotypic classes of the counter path SNP/INDEL alleles were also calculated.

RESULTS AND DISCUSSION

The identification of QTL and the establishment of numerous QTL haplotypes associated with varying degrees of the trait value are very important to initiate MAB for complex traits (He *et al.*, 2006; Ducrocq *et al.*, 2009; Li *et al.*, 2012). The *Pup1* QTL is molecularly

characterised (Ni *et al.*, 1998; Wissuwa *et al.*, 1998; Lang & Buu, 2006; Prasetyono *et al.*, 2010) in which associated haplotypes (Chin *et al.*, 2011; Tyagi *et al.*, 2012) and some of the underline genes are characterised (Chin *et al.*, 2011; Gamuyao *et al.*, 2012). However, many of the country and geographical location specific rice landraces and important varieties have not been included in genetic and physiological studies on PD tolerance. Many studies (Wissuwa & Ae, 2001; Chin *et al.*, 2011; Gamuyao *et al.*, 2012) have highlighted the importance of using tolerant landraces and hardy varieties to mine the interested alleles and haplotypes for MAB. Having the broader aim of establishing a comprehensive marker assisted rice breeding programme in Sri Lanka, a set of 30 rice genotypes containing landraces and improved varieties were characterised for PD tolerance (Aluwihare *et al.*, 2016).

Sequence alignment and analysis of polymorphism

The studied rice genotypes were characterised for 46 DNA markers developed for the *Pup1* region in previous studies (Heuer *et al.*, 2009; Chin *et al.*, 2010; Chin *et al.*, 2011). The *K20-1* STS region was selected for the present study and subjected to DNA sequence analysis. The *K20-1* region was chosen because of its co-dominant nature, easy and non-ambiguous amplification and the presence of significant polymorphism (Chin *et al.*, 2011). PCR amplification of genomic DNA using *K20-1* primers yielded a band with an approximate size of 240 bp. When *K20-1* DNA sequences (Table 2) of all 32 rice genotypes were aligned, a biallelic length polymorphism was detected where a 240 base long fragment was observed in 11 genotypes and a 243 base long fragment was observed in the other 21 genotypes. A total 11 nucleotide variations detected comprised 10 SNPs (nine transition and one transversion mutations) and one INDEL; and all of them were biallelic and consistent with the sequence information reported in Chin *et al.* (2011). Six SNPs were observed as reported in Chin *et al.* (2011) where either *Kasalath*-like or *Nipponbare*-like alleles were present (the SNPs found at 56th, 158th, 193rd, 198th, 210th and 212th positions). Novel SNP alleles were detected for the 64th, 70th, 106th and 209th base positions where *Kasalath*, *Nipponbare* and some of the Sri Lankan rice genotypes shared one SNP allele, and other Sri Lankan rice genotypes shared a different allele. The SNP found at the 209th position is particularly interesting as highly PD tolerant rice genotypes *Murungakayan*, *Suduheenati*, H-4, H-7 and H-10 have SNP allele 'A', whereas *Kasalath* and *Nipponbare* and other 26 Sri Lankan genotypes shared a common allele 'G' showing the presence of a transition mutation (Figure 1).

The three-base difference was due to the 'CAG' INDEL present in the position between 204th to 206th nucleotides. According to Chin *et al.* (2011), this INDEL is present in *Kasalath* and significantly associated with PD

tolerance. Accordingly the same INDEL was detected in nine genotypes in the present study and according to Aluwihare *et al.* (2016) these genotypes are either tolerant or moderately tolerant to PD. However, it is

Figure 1: DNA sequence alignment diagram for K20-1 region of the *Pup1* locus for 32 rice genotypes. The alignment was obtained using Clustal Omega software (www.ebi.ac.uk).

restriction sites *SduI* (*Bsp 1286I*) and *MseI* reported in Chin *et al.* (2011) are also observed in the present study in 10 tolerant or moderately tolerant genotypes (Figure 1).

Biallelic polymorphism was detected in all the SNPs and INDEL loci showing a limited level of heterozygosity present across the 32 rice genotypes tested. *K20-1* DNA sequences of the 32 rice genotypes (Figure 1) were translated to amino acid sequences with the relevant reading frame and only two amino acid changes were observed, in which valine to methionine mutation was at 19th position and phenylalanine to leucine mutation was at 53rd position (Figure 2).

two clusters were further divided into two sub clusters named C1 and CK (K stands for *Kasalath* type) at a similarity level of 45.23 %. The other cluster formed at 22.54 % similarity got further divided into two sub clusters at 55.28 % similarity. Out of these two clusters, one cluster was not further collapsed and labelled as CN (N stands for *Nipponbare* type) and the other cluster was collapsed at a similarity level of 68.37 % into two clear clusters that were labelled as C2 and C3. Unique haplotypes were observed for each cluster. The genotypes in clusters C2, C3 and CN contained the insertion ‘CAG’ and the genotypes in other clusters contained the deletion (i.e. ‘CAG’ was missing) (Figure 3).

Cluster CK designates the genotypes, which has 100 % similarity to the *K20-1* region of the *Kasalath*

genotype. The improved variety At 362, PD tolerant landraces *Sudubalawee*, *Marss*, and the moderately tolerant landrace *Rathuheenati* have identical *K20-1* regions to that of *Kasalath*. Cluster CN, the *Nipponbare* like cluster, contains two PD tolerant genotypes Bg 94-1 and *Rathel*, and moderately tolerant genotypes Bw 364, *Sudurusamba* and *Hondarawala*. However, no PD sensitive genotypes were observed within the CN cluster indicating the genetic complexity and the fact that *Nipponbare* type haplotype cannot always be used in MAB as the indicator haplotype of PD sensitivity. It also implies that a different PD sensitive mechanism could be present in Sri Lankan rice genotypes than that of *Nipponbare*. The cluster C1 contains three tolerant genotypes, Bg 403, *Kokuwellai* and *Kaluheenati*, and moderately tolerant genotypes Bg 250, At 353 and *Suwandel*. Cluster C2 contains only extremely tolerant H-4, H-7, H-10, *Murungakayan* and *Suduheenati* and

could be considered as the PD tolerant cluster in the studied germplasm. Cluster C3 has six moderately tolerant genotypes and four sensitive genotypes. All the sensitive genotypes detected in Aluwihare *et al.* (2016) are stacked within cluster C3 (Figure 3).

Pedigree analysis

Visualisation of pedigrees with respect to parental relationships, trait values and marker genotypes/haplotypes are very important in making decisions for breeding and genetic studies. Voorrips (2007) has developed a software called Pedimap, which could be employed efficiently and meaningfully to display such information to be easily used in breeding programmes. Pedimap based pedigree displays have been particularly used in Rosaceae research community (Rosyara *et al.*, 2013; Peace *et al.*, 2014) and can be used in rice research

Figure 3: The dendrogram constructed for 32 rice genotypes based on the ten SNPs and the INDEL mutation detected in the *K20-1* genomic region

and breeding as well. The *K20-1* sequences and their identical by descent with respect to the available pedigree relationship and overall PD tolerance level (Aluwihare *et al.*, 2016) were displayed using Pedimap software (Figure 4). Since pedigree relationships are not available for most of the genotypes within the 30 genotypes studied, the identical by descent was shown only for a few genotypes. The pedigree structure revealed the distribution of the degree of PD tolerance across all the genotypes. Brown colour in *Kasalath* represents maximum tolerance, and green colour in *Nipponbare*

represents maximum sensitivity. The haplotypes of the unrelated individuals in the illustration were assigned with unique colours by Pedimap. But the related individuals are shown with combined colours and the IBD of the haplotypes are also included. For example, At 354 has *Pokkali* and Bg 94-1 as parents but the *K20-1* region of At 354 has been inherited from *Pokkali*. At 353 might have received its haplotype from the unknown parent while Bg 94-1 is the male parent. H-4, a RIL from the cross between *Murungakayan* and *Marss*, is the highest tolerant variety according to Aluwihare

Figure 4: Visualisation of the PD tolerance score, *K20-1* SNP haplotypes and available pedigree relationships in 32 rice genotypes [redrawn using the output created by Pedimap software (Voorrips, 2007)]

et al. (2016), and has received its haplotype from the female parent *Murungakayan*. The genotypes At 353, H-10, H-7, Bg 357 and Bg 403, which have only one parent in the current set clearly displayed the parental origin of haplotypes. Although the female parent of H-7 and H-10 are not included in this study it was a sister RIL of H-4 named H-5 that should have received *K20-1* haplotype from the parental landrace *Murungakayan*. The genotypes Bg 357 and Bg 403 received their *K20-1* haplotypes from the paternal parent. The colour depiction of PD tolerance in 32 rice genotypes clearly explains the differential tolerance or sensitivity towards PD (Figure 4). Attempts are in place to convert the conventionally recorded pedigree relationships, trait values and available marker and sequence information as Pedimap based visual displays to facilitate rice breeding in Sri Lanka.

Association analysis

The association analyses conducted using TASSEL 5 software revealed that SNPs and INDEL alleles are associated with PD tolerance related traits. PIH measured under P_0 and P_{30} conditions was strongly associated with the SNP located at 209th base position ($p < 0.05$). In this SNP, when the allele 'A' was in homozygous condition (which could only be seen in some Sri Lankan rice genotypes), plants were taller than the plants which has homozygous 'G'. The NT was significantly associated with the eight SNPs and the INDEL ($p < 0.05$) (Table 3).

The only significant marker trait association for SDW was with the SNP at 209th position ($p < 0.05$). In homozygous 'A' class, the mean SDW was 23.82 g compared to mean SDW of 14.83 g in homozygous 'G' class ($p < 0.05$). Five SNPs and the INDEL were strongly associated with SPC and PUE. Only the SNP at 209th position was significantly associated with SPU ($p < 0.1$). The overall score of PD tolerance was associated with six SNPs (158th, 193rd, 198th, 209th, 210th and 212th positions) and the INDEL ($p < 0.05$) (Table 3).

In summary, the classical approach was followed in the *Pup1* QTL mapping studies in which the linkage relationship between markers and traits are considered (Wissuwa *et al.*, 1998; Wissuwa, 2003). However, no particular attention has been given to find the significant association between the SNPs and INDELs with the PD tolerance traits. In the present study, we report the association between PD tolerance traits and SNPs/INDEL of the *K20-1* region from a selected set of rice germplasm. It would be interesting to see an important core-collection of rice genotypes with varieties, mapping populations, unreleased breeding materials produced

Table 2: GenBank accession numbers of the *K20-1* DNA sequences of the rice genotypes

Name of the genotype	GenBank accession number
<i>Hondarawala</i>	KU174163
<i>Rathel</i>	KU174164
Bg 94-1	KU174174
<i>Sudurusamba</i>	KU174175
Bw 364	KU174176
Bg 352	KU174169
Pokkali	KU174166
Bg 357	KU174170
Bg 358	KU174171
Bg 450	KU174173
At 306	KU174178
Bg 300	KU174179
Ld 356	KU174182
At 354	KU174184
Bg 379-2	KU174186
<i>Murungakayan</i>	KU174158
<i>Suduheenati</i>	KU174159
H-4	KU174168
H-7	KU174177
H-10	KU174181
<i>Marss</i>	KU174157
<i>Sudubalawee</i>	KU174162
<i>Rathuheenati</i>	KU174165
At 362	KU174185
<i>Kaluheenati</i>	KU174160
<i>Suwandel</i>	KU174161
<i>Kokuwellai</i>	KU174167
At 353	KU174172
Bg 250	KU174180
Bg 403	KU174183

Source : <http://www.ncbi.nlm.nih.gov/>

from the original varieties and landraces are subjected to a meta-association analysis of the DNA sequence polymorphism and the traits. With the present study, we proved that *Kasalath* type *K20-1* haplotype is associated with PD tolerance but another haplotype, which is probably specific to Sri Lankan germplasm also confers a higher tolerance. We propose that these two haplotypes could be used in MAB of rice for PD tolerance. However, the reliance on *K20-1* PCR followed by *SduI* (*Bsp* 1286I) or *MseI* digestion to evaluate the polymorphism as a CAP maker is doubtful because the highest PD tolerant genotypes in the present study (the ones in C2 cluster) (Figure 3) do not have these palindromic sequences within the *K20-1* region. Therefore, we propose an alternative strategy of employing an allele specific oligonucleotide assay to detect the 'A' allele along with

the *SduI* (*Bsp* 1286I) and *MseI* digestion, to discriminate the tolerant genotypes from the sensitive ones in MAB or marker assisted seedling selection (MASS). These kinds of CAP markers and allele specific oligonucleotide assays are common in human, mouse and plant genetic and breeding studies (Saiki *et al.*, 1985; Hixson & Vernier, 1990; Gutierrez *et al.*, 2006). The establishment of simple and easier genotyping strategies of MAB is essential (Sofi & Rather, 2007; Pérez-de-Castro, 2012) and they should not be completely depending on the routine DNA sequencing and the hectic downstream bioinformatic analysis (Asins, 2002; Collard & Mackill, 2008). However, more in-depth sequence analyses of the entire *Pup1* QTL region with a larger germplasm panel would provide a deeper insight to mine more novel haplotypes that could be used in MAB for PD tolerance.

CONCLUSION

The DNA sequence analyses of *K20-1* region of the 30 rice genotypes revealed that PD tolerance is not only conferred by the *Kasalath* type sequence haplotype but also by another haplotype, which is present in the landrace *Murungakayan* and some of the old improved varieties in Sri Lanka. The DNA sequence polymorphisms within the *K20-1* region was further expanded by the current study compared to what is reported in Chin *et al.* (2011) envisaging the fact that meta-analysis of the DNA sequence polymorphism within the entire *Pup1* region of the important genotypes of the rice germplasm is essential to layout a precise and accurate haplotype based MAB strategy to produce PD tolerant rice genotypes for the diverse requirements in global rice industry.

Table 3: Association of PD tolerance related traits with the SNPs and the INDEL detected in *K20-1* STS region

Trait	Growing condition ^a	Locus (bp) ^b	Alleles ^c	Mean LL	Mean RR	GS	P value
PIH	P ₀ - GH-Maha	209	A/G	96.17	69.43	26.74	2.28E-04
	P ₃₀ - GH-Yala	209	A/G	77.81	53.17	24.64	5.37E-06
	P ₀ - F-Yala	209	A/G	82.10	62.95	19.15	0.033
	P ₃₀ - GH-Maha	209	A/G	91.91	73.24	18.67	0.003
	P ₀ - GH-Yala	209	A/G	88.04	58.72	29.32	6.70E-05
NT	P ₀ - F-Yala	106	A/G	2.28	3.20	-0.92	0.004
	P ₃₀ - GH-Yala	106	A/G	3.99	6.44	-2.45	0.021
	P ₀ - F-Yala	158	C/T	3.20	2.28	0.92	0.004
	P ₃₀ - GH-Yala	158	C/T	6.44	3.99	2.45	0.021
	P ₀ - F-Yala	193	C/T	2.28	3.20	-0.92	0.004
	P ₃₀ - GH-Yala	193	C/T	3.99	6.44	-2.45	0.021
	P ₀ - F-Yala	198	C/T	2.28	3.20	-0.92	0.004
	P ₃₀ - GH-Yala	198	C/T	3.99	6.44	-2.45	0.021
	P ₀ - F-Yala	204-206	+ / - ^d	2.28	3.20	-0.92	0.004
	P ₃₀ - GH-Yala	204-206	+ / - ^d	3.99	6.44	-2.45	0.021
	P ₀ - F-Yala	210	C/T	3.20	2.28	0.92	0.004
	P ₃₀ - GH-Yala	210	C/T	6.44	3.99	2.45	0.021
	P ₀ - F-Yala	212	A/G	3.20	2.28	0.92	0.004
	P ₃₀ - GH-Yala	212	A/G	6.44	3.99	2.45	0.021
	P ₀ - F-Yala	64	C/G	3.20	2.28	0.92	0.004
	P ₃₀ - GH-Yala	64	C/G	6.44	3.99	2.45	0.021
	P ₀ - F-Yala	70	C/T	3.20	2.28	0.92	0.004
	P ₃₀ - GH-Yala	70	C/T	6.44	3.99	2.45	0.021
SDW	P ₀ - GH-Maha	209	A/G	23.82	14.83	9.49	3.54E-04
SPC	P ₀ - F-Maha	158	C/T	0.54	0.68	-0.14	0.071
	P ₀ - F-Maha	193	C/T	0.68	0.54	0.14	0.071
	P ₀ - F-Maha	198	C/T	0.68	0.54	0.14	0.071
	P ₀ - F-Maha	204-206	+ / - ^d	0.68	0.54	0.14	0.071
	P ₀ - F-Maha	210	C/T	0.54	0.68	-0.14	0.071
SPU	P ₀ - F-Maha	212	A/G	0.54	0.68	-0.14	0.071
	P ₀ - GH-Maha	209	A/G	12.96	9.76	3.19	0.059
	P ₃₀ - GH-Yala	209	A/G	19.34	14.83	4.50	0.064

continued -

continued from page 422

Trait	Growing condition ^a	Locus (bp) ^b	Alleles ^c	Mean LL	Mean RR	GS	P value
PUE	P ₀ - F-Maha	158	C/T	1.94	1.54	0.40	0.058
	P ₀ - F-Maha	193	C/T	1.54	1.94	-0.40	0.058
	P ₀ - F-Maha	198	C/T	1.54	1.94	-0.40	0.058
	P ₀ - F-Maha	204-206	+ / - ^d	1.55	1.94	-0.39	0.058
	P ₀ - F-Maha	210	C/T	1.94	1.55	0.39	0.058
	P ₀ - F-Maha	212	A/G	1.94	1.54	0.40	0.058
PDT	P ₀ - GH-Maha	158	C/T	2.63	2.10	0.53	0.044
	P ₀ - GH-Maha	193	C/T	2.10	2.64	-0.54	0.044
	P ₀ - GH-Maha	198	C/T	2.10	2.64	-0.54	0.044
	P ₀ - GH-Maha	204-206	+ / - ^d	2.10	2.64	-0.54	0.044
	P ₀ - GH-Maha	209	A/G	3.00	2.14	0.86	0.013
	P ₀ - GH-Maha	210	C/T	2.64	2.10	0.54	0.044
	P ₀ - GH-Maha	212	A/G	2.64	2.10	0.54	0.044

^a The growing condition represent the P level supplied (P₀: 1 mg of P in 1 kg of soil and P₃₀: 30 mg of P₂O₅ was added to 1 kg of Ultisol soil) and greenhouse (GH) or field (F) conditions used. *Yala* and *Maha* represent the two general cropping seasons in Sri Lanka

^b The base position within the K20-1 STS region which has the total length of 240 or 243 bp (Figure 1)

^c The SNP or INDEL alleles detected in each locus. Only biallelic SNPs are detected

^d CAG insertion can be seen in 11 rice genotypes at the position 204 bp to 206 bp

Mean LL represent the mean phenotype when the allele shown in the left side of the SNP column are in homozygous condition. Mean RR represent the mean phenotype when the allele shown in the right side of the SNP column are in homozygous condition. GS designates the gain of selection which is calculated by subtracting the mean RR by mean LL. P value indicates the probability level for significant mean differences calculated using Tassel 5.0 software. Alleles shown with white colour font in black background are present only in Sri Lankan rice germplasm. Alleles shown in gray background are present in both *Kasalath*, *Nipponbare* and some of the Sri Lankan rice genotypes. Alleles underlined are present in *Kasalath* and some of the Sri Lankan genotypes. Alleles not underlined are present in *Nipponbare* and some of the Sri Lankan genotypes. SDW: shoot dry weight (grams per pot); SPC: shoot P concentration [amount of P (mg) in 1 gram of shoot dry]; SPU: shoot P uptake (mg P per pot); PUE: P utilisation efficiency (biomass produced per unit P accumulated in shoot tissue); PDT: P deficiency tolerance score assigned in Aluwihare *et al.* (2016).

Acknowledgement

The authors wish to acknowledge the National Research Council (NRC), Sri Lanka (Grant No: NRC 11-087) for financial assistance.

REFERENCES

- Aluwihare Y.C., Ishan M., Chamikara M.D.M., Weebadde C.K., Sirisena D.N., Samarasinghe W.L.G. & Sooriyapathirana S.D.S.S. (2016). Characterization and selection of phosphorus deficiency tolerant rice genotypes in Sri Lanka. *Rice Science* **23**(4): 184 – 195.
DOI: <https://doi.org/10.1016/j.rsci.2015.10.001>
- Asins M. (2002). Present and future of quantitative trait locus analysis in plant breeding. *Plant Breeding* **121**: 281 – 291.
DOI: <https://doi.org/10.1046/j.1439-0523.2002.730285.x>
- Bennett E.M., Carpenter S.R. & Caraco N.F. (2001). Human impact on erodable phosphorus and eutrophication: a global perspective increasing accumulation of phosphorus in soil threatens rivers, lakes, and coastal oceans with eutrophication. *BioScience* **51**: 227 – 234.
- Bradbury P.J., Zhang Z., Kroon D.E., Casstevens T.M., Ramdoss Y. & Buckler E.S. (2007). TASSEL 5: software for association mapping of complex traits in diverse samples. *Bioinformatics* **23**: 2633 – 2635.
DOI: <https://doi.org/10.1093/bioinformatics/btm308>
- Chandrajith R., Seneviratna S., Wickramaarachchi K., Attanayake T., Aturaliya T.N.C. & Dissanayake C.B. (2010). Natural radionuclides and trace elements in rice field soils in relation to fertilizer application: study of a chronic kidney disease area in Sri Lanka. *Environmental Earth Sciences* **60**(1): 193 – 201.
DOI: <https://doi.org/10.1007/s12665-009-0179-1>
- Chin J.H., Gamuyao R., Dalid C., Bustamam M., Prasetyono J., Moeljopawiro S., Wissuwa M. & Heuer S. (2011). Developing rice with high yield under phosphorus deficiency: *Pup1* sequence to application. *Plant Physiology* **156**(3): 1202 – 1216.
DOI: <https://doi.org/10.1104/pp.111.175471>
- Chin J.H., Lu X., Haefele H.M., Gamuyao R., Ismail A.M., Heuer S. & Wissuwa M. (2010). Development and application of gene-based markers for the major rice QTL phosphorus uptake 1. *Theoretical and Applied Genetics*

- 120(6): 1073 – 1086.
DOI: <https://doi.org/10.1007/s00122-009-1235-7>
8. Collard B.C.Y. & Mackill D.J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences* **363**: 557 – 572.
DOI: <https://doi.org/10.1098/rstb.2007.2170>
 9. Cordell D., Drangert J.O. & White S. (2009). The story of phosphorus: global food security and food for thought. *Global Environmental Change* **19**: 292 – 305.
DOI: <https://doi.org/10.1016/j.gloenvcha.2008.10.009>
 10. Department of Agriculture (DoA) (2006a). *Climate of Rice Growing Regions in Sri Lanka*. Department of Agriculture, Sri Lanka. Available at <http://www.agridept.gov.lk/>. Accessed 17 October 2016.
 11. Department of Agriculture (DoA) (2006b). *Recommended Varieties Released List*. Department of Agriculture, Sri Lanka. Available at <http://www.agridept.gov.lk/>. Accessed 17 October 2016.
 12. Ducrocq S., Giauffret C., Madur D., Combes V., Dumas F., Jouanne S., Coubriche D., Jamin P., Moreau L. & Charcosset A. (2009). Fine mapping and haplotype structure analysis of a major flowering time quantitative trait locus on maize chromosome 10. *Genetics* **183**(4): 1555 – 1563.
DOI: <https://doi.org/10.1534/genetics.109.106922>
 13. Fageria N.K., Filho M.P.B. & Carvalho J.R.P. (1982). Response of upland rice to phosphorus fertilization on an oxisol of central Brazil. *Agronomy Journal* **74**: 51 – 56.
 14. Fageria N.K., Wright R.J. & Baligar V.C. (1988). Rice cultivar evaluation for phosphorus use efficiency. *Plant and Soil Science* **111**(1): 105 – 109.
DOI: <https://doi.org/10.1007/BF02182043>
 15. Gamuyao R., Chin J.H., Pariasca-Tanaka J., Pesaresi P., Catausan S., Dalid C., Slamet-Loedin I., Tecson-Mendoza E.M., Wissuwa M. & Heuer S. (2012). The protein kinase *Pstol1* from traditional rice confers tolerance of phosphorus deficiency. *Nature* **488**: 535 – 539.
DOI: <https://doi.org/10.1038/nature11346>
 16. Gunes A., Inal A., Alpaslan M. & Cakmak I. (2006). Genotypic variation in phosphorus efficiency between wheat cultivars grown under greenhouse and field conditions. *Soil Science and Plant Nutrition* **52**(4): 470 – 478.
DOI: <https://doi.org/10.1111/j.1747-0765.2006.00068.x>
 17. Gutierrez N., Avila C.M., Duc G., Marget P., Suso M.J., Moreno M.T. & Torres A.M. (2006). CAPs markers to assist selection for low vicine and convicine contents in faba bean (*Vicia faba* L.). *Theoretical and Applied Genetics* **114**(1): 59 – 66.
DOI: <https://doi.org/10.1007/s00122-006-0410-3>
 18. Hanson W.C. (1950). The photometric determination of phosphorus in fertilizers using the phosphovanadomolybdate complex. *Journal of the Science of Food and Agriculture* **1**: 172 – 173.
DOI: <https://doi.org/10.1002/jsfa.2740010604>
 19. He G. et al. (11 authors) (2006). Haplotype variation in structure and expression of a gene cluster associated with a quantitative trait locus for improved yield in rice. *Genome Research* **16**(5): 618 – 626.
DOI: <https://doi.org/10.1101/gr.4814006>
 20. Hedley M.J., Kirk G.J.D. & Santos M.B. (1994). Phosphorus deficiency and the forms of soil phosphorus utilized by upland rice cultivars. *Plant and Soil* **158**: 53 – 62.
DOI: <https://doi.org/10.1007/BF00007917>
 21. Heuer S. et al. (11 authors) (2009). Comparative sequence analyses of the major quantitative trait locus phosphorus uptake 1 (*Pup1*) reveal a complex genetic structure. *Plant Biotechnology Journal* **7**(5): 456 – 471.
DOI: <https://doi.org/10.1111/j.1467-7652.2009.00415.x>
 22. Hixson J.E. & Vernier D.T. (1990). Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *The Journal of Lipid Research* **31**(3): 545 – 548.
 23. Ismail A.M., Heuer S., Thomson M.J. & Wissuwa M. (2007). Genetic and genomic approaches to develop rice germplasm for problem soils. *Plant Molecular Biology* **65**(4): 547 – 570.
DOI: <https://doi.org/10.1007/s11103-007-9215-2>
 24. Jiao W., Chen W., Chang A.C. & Page A.L. (2012). Environmental risks of trace elements associated with long-term phosphate fertilizers applications: a review. *Environmental Pollution* **168**: 44 – 53.
DOI: <https://doi.org/10.1016/j.envpol.2012.03.052>
 25. Kirk G.J.D., George T., Courtois B. & Senadhira D. (1998). Opportunities to improve phosphorus efficiency and soil fertility in rain fed lowland and upland rice ecosystems. *Field Crops Research* **56**: 73 – 92.
 26. Kumaragamage D. & Indraratne S. (2011). Systemic approach to diagnosing fertility problems in soils of Sri Lanka. *Communications in Soil Science and Plant Analysis* **42**(22): 2699 – 2715.
DOI: <https://doi.org/10.1080/00103624.2011.622818>
 27. Lang N.T. & Buu B.C. (2006). Mapping QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Omonrice* **14**: 1 – 9.
 28. Li L., Qiu X., Li X., Wang S. & Lian X. (2009). The expression profile of genes in rice roots under low phosphorus stress. *Science in China Series C: Life Sciences* **52**(11): 1055 – 1064.
DOI: <https://doi.org/10.1007/s11427-009-0137-x>
 29. Li Y. et al. (11 authors) (2012). Comparative LD mapping using single SNPs and haplotypes identifies QTL for plant height and biomass as secondary traits of drought tolerance in maize. *Molecular Breeding* **30**(1): 407 – 418.
DOI: <https://doi.org/10.1007/s11032-011-9631-5>
 30. Ni J.J., Wu P., Senadhira D. & Huang N. (1998). Mapping QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **97**: 1361 – 1369.
DOI: <https://doi.org/10.1007/s001220051030>
 31. Pariasca-Tanaka J., Chin J.H., Dramé K.N., Dalid C., Heuer S. & Wissuwa M. (2014). A novel allele of the P-starvation tolerance gene *OsPSTOL1* from African rice (*Oryza glaberrima* Steud) and its distribution in the genus *Oryza*. *Theoretical and Applied Genetics* **127**: 1387 – 1398.
DOI: <https://doi.org/10.1007/s00122-014-2306-y>
 32. Peace C.P., Luby J.J., van de Wag W.E., Bink M.C.A.M. & Iezzoni A.F. (2014). A strategy for developing

- representative germplasm sets for systematic QTL validation, demonstrated for apple, peach, and sweet cherry. *Tree Genetics and Genomes* **10**(6): 1679 – 1694.
DOI: <https://doi.org/10.1007/s11295-014-0788-z>
33. Pérez-de-Castro A.M., Vilanova S., Canñizares J., Pascual L., Blanca J.M., Diez M.J., Prohens J. & Picó B. (2012). Application of genomic tools in plant breeding. *Current Genomics* **13**(3): 179 – 195.
DOI: <https://doi.org/10.2174/138920212800543084>
 34. Prasetyono J., Aswidinnoor H., Moeljopawiro S., Sopandie D. & Bustamam M. (2010). Identification of polymorphic markers for breeding of rice tolerant to phosphorus deficiency. *Indonesian Journal of Agriculture* **3**(1): 1 – 8.
 35. Rose T.J. & Wissuwa M. (2012). Rethinking internal phosphorus utilization efficiency: a new approach is needed to improve PUE in grain crops. *Advances in Agronomy* **116**: 186 – 211.
DOI: <https://doi.org/10.1016/B978-0-12-394277-7.00005-1>
 36. Rosyara U.R., Bink M.C.A.M., van de Weg E., Zhang G., Wang D., Sebolt A., Dirlwanger E., Quero-Garcia J., Schuster M. & Iezzoni A.F. (2013). Fruit size QTL identification and the prediction of parental QTL genotypes and breeding values in multiple pedigreed populations of sweet cherry. *Molecular Breeding* **32**(4): 875 – 887.
DOI: <https://doi.org/10.1007/s11032-013-9916-y>
 37. Saiki R.K., Scharf S., Faloona F., Mullis K.B., Horn G.T., Erlich H.A. & Arnheim N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**(4732): 1350 – 1354.
DOI: <https://doi.org/10.1126/science.2999980>
 38. Sofi P. & Rather A.G. (2007). QTL analysis in rice improvement: concept, methodology and application. *Biotechnology* **6**(1): 1 – 13.
DOI: <https://doi.org/10.3923/biotech.2007.1.13>
 39. Tyagi W., Rai M. & Dohling A. (2012). Haplotype analysis for *Pup1* locus in rice genotypes of North Eastern and Eastern India to identify suitable donors tolerant to low phosphorus. *SABRAO Journal of Breeding and Genetics* **44**(2): 398 – 405.
 40. Voorrips R.E. (2007). *Pedimap*: software for visualization of genetic and phenotypic data in pedigrees. Plant Research International, Wageningen, The Netherlands.
 41. Wissuwa M. (2003). How do plants achieve tolerance to phosphorus deficiency: small causes with big effects. *Plant Physiology* **133**: 1947 – 1958.
DOI: <https://doi.org/10.1104/pp.103.029306>
 42. Wissuwa M. (2005). Combining a modelling with a genetic approach in establishing associations between genetic and physiological effects in relation to phosphorus uptake. *Plant and Soil* **269**: 57 – 68.
DOI: <https://doi.org/10.1007/s11104-004-2026-1>
 43. Wissuwa M. & Ae N. (2001). Further characterization of two QTLs that increase phosphorus uptake of rice (*Oryza sativa* L.) under phosphorus deficiency. *Plant and Soil* **237**: 275 – 286.
DOI: <https://doi.org/10.1023/A:1013385620875>
 44. Wissuwa M., Gamat G. & Ismail A.M. (2005). Is root growth under phosphorus deficiency affected by source or sink limitations? *Journal of Experimental Botany* **56**(417): 1943 – 1950.
DOI: <https://doi.org/10.1093/jxb/eri189>
 45. 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. *Theoretical and Applied Genetics* **105**: 890 – 897.
DOI: <https://doi.org/10.1007/s00122-002-1051-9>
 46. Wissuwa M., Yano M. & Ae N. (1998). Mapping of QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **97**(5): 777 – 783.
DOI: <https://doi.org/10.1007/s001220050955>
 47. 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 Physiology* **138**: 2087 – 2096.
DOI: <https://doi.org/10.1104/pp.105.063115>