

RESEARCH ARTICLE

Validation of *K46*, a *Pup1*-linked marker, using a selection of Sri Lankan rice (*Oryza sativa* L.) germplasm for marker assisted selection towards phosphorous deficiency tolerance

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ABSTRACT

Phosphorous deficiency (PD) tolerance is a pivotal trait that is advantageous if present in modern day high performing rice varieties. However most of the frequently grown mega rice varieties lack this trait leading to expensive application of artificial phosphate fertilizer results secondary consequences such as environmental pollution and higher cost of production. Marker assisted breeding (MAB) for PD tolerance in rice is often hailed as the pragmatic solution to tackle this problem. The genetic basis of PD tolerance in rice has been dissected using a wide cross made between PD tolerant rice landrace *Kasalath* and a sensitive landrace *Nipponbare*. A major QTL known as *Pup1* has been identified, molecularly characterized and underlying polymorphisms were detected. A major INDEL region within *Pup1* QTL within *Kasalath* background is conferred to control the PD tolerance which is genetically 'null' in *Nipponbare* background. The DNA marker *K46* which is present within *Pup1* has been developed to identify this INDEL region in *Kasalath* like genotypes. The PD tolerance in Sri Lankan rice germplasm has been recently studied using a core panel of rice genotypes that are important in rice breeding programs. It is important to figure out the genomic landscape of the INDEL region of *Pup1* QTL in these Sri Lankan rice genotypes. Therefore the present study was conducted to characterize the *K46* DNA marker locus of the previously characterized for PD tolerance rice cultivars in Sri Lanka. The *K46* specific primer-pair was assayed across 30 selected Sri Lankan cultivars and the PCR products were sequenced. The resulted DNA sequences were aligned with the *Kasalath* reference sequence for *K46* locus. Further cluster analysis of the identified SNPs resulted four distinct haplotypes in which nine cultivars were grouped with *Kasalath* like haplotype, two unique haplotypes and the null haplotype. However, there is no strong association between the haploype class and the PD tolerance score of the cultivars implying the potentially novel PD tolerance mechanisms that require further studies.

Keywords: Phosphorous deficiency tolerance, *Oryza sativa*, *K46* marker, marker assisted selection, rice germplasm in Sri Lanka.

INTRODUCTION

Since green revolution, profitable rice farming is believed to be entirely dependent on the application of artificial fertilizer. The higher and expedited responding of rice cultivars to fertilizer application was considered as a key trait (MacDonald *et al.*, 2011). However, currently it is believed that the application of fertilizer itself cannot increase the rice production due to many reasons such as fixing nutrients by the soil systems and higher cost associated with the fertilizer inputs (Arai and Sparks, 2007). Moreover prolonged and handful applications of fertilizer could dramatically pollute the environment (Cordell *et al.*, 2009) and also contribute to the health hazards (Chandrajith *et al.*, 2010). Phosphorous (P) is the most problematic nutrient in rice growing soils. Phosphorous gets

fixed by Fe³⁺ and Al³⁺ ions present in the soils and thereby becoming limitedly available to the plants (Shen *et al.*, 2011; Holford and Mattingly, 1976; Bielecki, 1973). In fact most of the rice growing soils lack the optimum levels of P for growth and development of rice (Fairhurst *et al.*, 1999). Because of these reasons farmers tend to apply more P fertilizer, but consequently they pollute the environment and increase the cost of production. Governments of the developing countries spend lot of money on fertilizer imports and in Sri Lanka it approximately accounts for 0.3 billion US dollars annually (Central Bank, Sri Lanka, 2014). The situation is further aggravated when government funded fertilizer subsidies are placed due to numerous socio-economic and political reasons (Cordell *et al.*, 2009). Organic rice growers also face difficulties as the popular organic manure such

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as crop stubbles and straw lack sufficient levels of P (Sirisena and Wanninayake, 2014; Kumaragamage and Indraratne, 2011). Therefore, an alternative strategy to overcome the P fertilizer led crisis is required.

To solve this problem, the plant research community considers breeding of P deficiency (PD) tolerant rice varieties as the most promising solution (Cordell *et al.*, 2009; Wissuwa and Ae, 2001a; Rose *et al.*, 2011). The PD tolerance is found to be quantitatively inherited with a significantly higher level of heterosis ranging from -60.6 to 148.6 (Majumder *et al.*, 1989). It was an encouraging sign for the rice geneticists and 17 years ago detailed quantitative genetic studies were begun to dissect the underlying genetics of PD tolerance in rice (Chin *et al.*, 2011; Wissuwa *et al.*, 1998; Doerge and Churchill, 1996). A major quantitative trait locus (QTL) controlling PD tolerance was identified on the rice chromosome 12 using recombinant inbred lines (RILs) generated from a cross between a PD tolerant landrace *Kasalath* and sensitive landrace *Nipponbare* (Wissuwa and Ae, 2001b; Wissuwa *et al.*, 2002). Later this QTL was fine mapped and labeled as *Pup1* (Wissuwa *et al.*, 1998; Heuer *et al.*, 2009; Chin *et al.*, 2010; Chin *et al.*, 2011). The *Pup1* QTL in *Kasalath* which is tolerant to PD, is 278 kb in length and *Nipponbare* which is sensitive to PD is only 145 kb in length (Gamuyao *et al.*, 2012; Heuer *et al.*, 2009) due to an INDEL. Thus, this major INDEL in the *Pup1* locus is a key determinant in conferring PD tolerance in *Kasalath*-like rice germplasm (Chin *et al.*, 2010).

The molecular markers have been developed to introgress the *Pup1* alleles conferring PD tolerance into PD sensitive rice genotypes (Chin *et al.*, 2010; Heuer *et al.*, 2009; Chin *et al.*, 2011). The DNA marker *K46*, present in the major INDEL within *Pup1* QTL and produces a 523 bp allele in *Kasalath* genetic background and a null allele (i.e. no band) in *Nipponbare* background (Chin *et al.*, 2010).

However, the presence of *K46* locus is not validated across a diverse rice germplasm collection to confirm it as a diagnostic marker for PD tolerance. In Sri Lanka, although MAB is still in its infancy, PD tolerance in rice and the importance of using genomic information in breeding is highlighted (Kottarachchi and Wijesekara, 2013). An important set of landraces and improved varieties (here in after collectively referred to as a panel of cultivars) were characterized for PD tolerance based on a three-tier indexing system (Aluwihare *et al.*, 2015). However, no attempts are reported in characterizing the *Pup1* locus to lay a foundation towards the MAB for PD tolerance. Therefore, the present study was conducted to characterize a set of rice cultivars previously evaluated for PD tolerance in Aluwihare *et al.*, (2015) for the DNA

sequence polymorphism in *K46* marker locus to lay a foundation for MAB to produce better performing rice varieties under low P conditions.

MATERIALS AND METHODS

Materials and methods

A set of 30 rice cultivars were obtained from Rice Research and Development Institute (RRDI), Bathalagoda (Bg), Sri Lanka (Appendix 1). Seeds were germinated, immature leaf samples were collected from young seedlings and DNA was extracted using the Dneasy Plant Minikit® (Qiagen, Solna, Sweden). Extracted DNA was confirmed for the successful PCR amplification using *matK*, a universal plant DNA barcoding primer pair (Hollingsworth *et al.*, 2011). Then PCR amplification was carried out using *K46* specific-primer pair (P_F: 5'TGAGATAGCCGTCAAGATGCT and P_R: 5'AAGGACCACCATTCCATAGC; Chin *et al.*, 2010). The PCR conditions were as follows, initial denaturation at 94 °C for min, 35 cycles of 94 °C for 0.5 min, 57 °C for 1.5 min, 72 °C for 2 min and final extension at 72 °C for 10 min. The PCR products were resolved in 1.5 % agarose gel electrophoresis and purified using a PCR clean-up system (Promega Corporation, Madison, WI, USA). The purified products were subjected to DNA sequencing using ABI 3500 series Genetic Analyzer (Applied Biosystems®, USA)

Data Analysis

The *K46* locus sequence of *Kasalath* (GenBank accession no: AB458444.1) was retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank/) and was used as the reference sequence. The amplified *K46* DNA sequences of rice cultivars were aligned with the reference sequence using ClustalW software (www.ebi.ac.uk). The DNA polymorphism of *K46* locus among the selected rice cultivars were subjected to cluster analysis by using pair-wise Simple Matching Coefficients (Webb and Copey, 2011) calculated based on the shared single nucleotide polymorphisms (SNPs). A dendrogram was constructed by unweighted pair-wise group method using average (UPGMA) based hierarchical clustering (Nei and Kumar, 2000). The haplotypes and PD tolerance scores of the rice cultivars (Aluwihare *et al.*, 2015) were subjected to association analysis using the Statistical Package SPSS 16 (SPSS Inc., 2007; Landau and Everitt, 2004).

RESULTS

PCR amplification of *K46* locus produced a distinct band having an approximate size of 500 bp for 15

out of 30 rice cultivars (Figure 1). When these PCR products were subjected to DNA sequencing, the exact length was revealed as 523 bp which is compatible with the *K46* locus in *Kasalath* (Chin *et al.*, 2010). A total of six SNPs were detected at the 47th, 225th, 226th, 247th, 339th and 420th positions along the 523 bp region, indicating the overall degree of polymorphism was 1.15 %. Out of the six SNPs, four transition and two transversion mutations were detected. All six SNPs were bi-allelic and were distributed along the locus without any apparent pattern (Figure 2).

The DNA sequence polymorphism in the *K46* locus of 16 cultivars including *Kasalath* revealed three clusters (*i.e.* three distinct haplotypes) at 84.33 % of sequence similarity. Cluster one (C1) contained nine rice cultivars including *Kasalath* and therefore, was referred to as the *Kasalath*-like cluster, cluster two (C2) included two cultivars *Pokkali* and At 354 and cluster three (C3) contained five rice cultivars. The SMC based UPGMA clustering revealed that the *Kasalath*-like cluster (C1) was 84.33 % similar to C2 and C3 was only 58.33 % similar to the other two clusters (Figure 3). The set of 15 cultivars in which the priming site of *K46* locus is absent was considered as the C4. When the overall score of PD tolerance of the cultivars was compared with the *K46* haplotype, it was found that, PD tolerance score of the cultivars were not significantly associated with the *K46* haplotypes (Table 1; $p < 0.05$).

DISCUSSION

Molecular breeding (*i.e.* MAS or MAB) is a theoretically sound and logical timely paradigm shift from the conventional breeding (Collard and Mackill, 2008; Xu and Crouch, 2008). However, complex polygenic traits provide a great deal of skepticism in molecular breeding when it is put into the practice as they are also controlled by the

environment (Xu and Crouch, 2008). Although PD tolerance is an important trait in rice breeding, it is a complex trait controlled by genetic and environmental factors. The characteristic feature of plants under P deprived conditions is the enhancement of the expression of specific genes to uptake more Pi from roots in P deficient soil conditions. *Pup1* has been identified as the major QTL for P deficiency tolerance in rice causes enhanced Pi uptake under P deprived conditions (Wissuwa *et al.*, 2002). The DNA marker *K46* was developed along with another set of markers to specifically target the INDEL region of the *Pup1* in *Kasalath*-like rice genomic background and to introgress the *Pup1* QTL effectively into the missing backgrounds (Chin *et al.*, 2010; Chin *et al.*, 2011, Pariasca-Tanaka *et al.*, 2014). However, the present study has clearly indicated that the employment of *K46* in molecular breeding for improved PD tolerance is quite inefficient (Table 1). Marker validations for country specific programs is a prerequisite before launching MAB into routine practice given the nature of higher diversity of rice germplasm (Ni *et al.*, 2002; Gao, 2003) and utilization of a narrow set of genotypes from the global rice germplasm for initial discovery of markers.

Although the major INDEL region where *K46* locus is missing in the rice landrace *Nipponbare*, there could be other rice genotypes which are significantly PD tolerant yet do not contain the INDEL region. In the present study there are two landraces *Murungakayan* and *Suduheenati* and three improved rice varieties H-4, H-7 and Bg 94-1 which are tolerant to PD (Aluwihare *et al.*, 2015) (Appendix 1) but possess a null allele for the *K46* locus (*i.e.* INDEL is missing). In addition, two landraces *Sudurusamba* and *Hondarawala* and six improved varieties which are classified as moderately tolerant (Aluwihare *et al.*, 2015) (Appendix 1) also do not contain the *K46* locus.

Table 1 The two-way contingency table depicting the association analysis between the *K46* haplotype and the degree of PD tolerance

	Cluster	PD tolerance (Aluwihare <i>et al.</i> , 2015)		
		1	2	3
<i>K46</i> haplotype	Null (C4)	2	8	6
	<i>Kasalath</i> type (C1)	1	2	4
	C2	0	2	3
	C3	1	1	0

Pearson Chi Square: 4.867 (not significant at $p < 0.05$).

Cramer's V Coefficient: 0.285 (not significant at $p < 0.05$).

Number of cultivars are shown in the each for respective categories

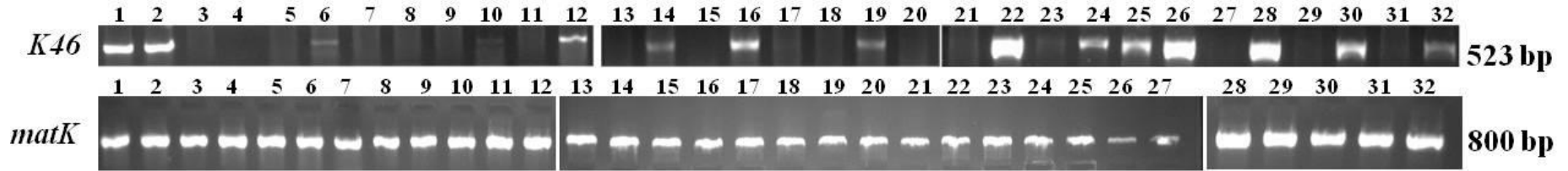


Figure 1 The *K46* DNA marker polymorphism in rice cultivars. The bands obtained for *matK* DNA barcoding locus is included to show the quality of DNA. 1: At 362, 2: *Pokkali*, 3: Bg 358, 4: Bg 450, 5: *Sudurusamba*, 6: Bg 379-2, 7: *Hondarawala*, 8: Bg 352, 9: Bg 250, 10: Bw 364, 11: At 353, 12: *Suwandel*, 13: Ld 356, 14: *Rathuheenati*, 15: Bg 357, 16: At 306, 17: At 354, 18: Bg 300, 19: *Kasalath*, 20: *Nipponbare*, 21: H-4, 22: *Marss*, 23: *Suduheenati*, 24: H-10, 25: *Rathel*, 26: *Kaluheenati*, 27: *Murungakayan*, 28: *Kokuwellai*, 29: H-7, 30: *Sudubalawee*, 31: Bg 94-1, and 32: Bg 403. As the positive and negative controls *Kasalath* (19) and *Nipponbare* (20) were included.

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Suwandel      TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT 120
Rathuheenati TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
H-10          TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Kokuwellai  TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Bg 403       TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
At 306       TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Bg 379-2    TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Rathel     TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Marss     TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Kaluheenati TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Sudubalawee TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Bw 364      TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Pokkali   TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
At 354      TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
At 362     TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
Kasalath  TGAGATAGCCGTC AAGATGCTAAAGGATACCAAGGGTGATGGGGAGGAATTCATAAATGAGGTTGCTGGCATTAGTAAAACCTTCTCATATCAATGTTGTTAACCTTCTAGGTTTTTCCCT
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Suwandel   TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCAC TTGATAGATATTC TTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTAGGATAGACTGTTCAA 240
Rathuheenati TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCAC TTGATAGATATTC TTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTAGGATAGACTGTTCAA
H-10        TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCAC TTGATAGATATTC TTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTAGGATAGACTGTTCAA
Kokuwellai TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCAC TTGATAGATATTC TTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTAGGATAGACTGTTCAA
Bg 403     TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCAC TTGATAGATATTC TTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTAGGATAGACTGTTCAA
    
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At 306 TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Bg 379-2 TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Rathel TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAAATAACACCCTGAGCTGGGATAGACTGTTCAA
Marss TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Kaluheenati TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Sudubalawee TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Bw 364 TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Pokkali TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
At 354 TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
At 362 TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA
Kasalath TCAAGGGTCAAAAAGAGCTCTGATCTATGAGTACATGCCCAATGGTTCACCTTGATAGATATTTCTTTTGGCGATAGCTCTGTCCAAGGAGATAACACCCTGAGCTGGGATAGACTGTTCAA

Suwandel TATTATCGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGATCAAGATTTCTGTCCAAAGAT 360
Rathuheenati TATTATCGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGATCAAGATTTCTGTCCAAAGAT
H-10 TATTATCGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGATCAAGATTTCTGTCCAAAGAT
Kokuwellai TATTATCGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGATCAAGATTTCTGTCCAAAGAT
Bg 403 TATTATCGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGATCAAGATTTCTGTCCAAAGAT
At 306 TGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Bg 379-2 TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Rathel TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Marss TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Kaluheenati TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Sudubalawee TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Bw 364 TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Pokkali TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
At 354 TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
At 362 TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT
Kasalath TATTATGTCGGGATTGCTCGAGGGCTGGAGTATCTCCACTGTCTATTGCAACATTCGCATTGTGCATTTTGATATCAAACCTCAAAACATTTCTACTGGCTCAAGATTTCTGTCCAAAGAT

Suwandel CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC 480
Rathuheenati CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
H-10 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Kokuwellai CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Bg 403 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
At 306 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Bg 379-2 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Rathel CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Marss CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Kaluheenati CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Sudubalawee CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Bw 364 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Pokkali CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
At 354 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
At 362 CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC
Kasalath CTCTGATTTTGGCCTGTCAAATTTGTGCCATCTAAAGGAGAGCAGAATTTTCGATCAACGCACTAAGAGGAACACCTGGCTACATTGCACCTGAAGTGTTTTCCAGGCAGTATGGATCTGC

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Suwandel          CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT 523
Rathuheenati     CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
H-10                CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Kokuwellai      CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Bg 403              CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
At 306              CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Bg 379-2            CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Rathel           CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Marss            CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Kaluheenati     CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Sudubalawee    CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Bw 364              CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Pokkali         CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
At 354              CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
At 362              CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
Kasalath        CAGCAGCAAATCTGATGTCTACAGCTATGGAATGGTGGTCCTT
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Figure 2 DNA Sequence alignment of *K46* region of the *Pup1* locus for 16 genotypes. The symbol ‘*’ indicates monomorphic nucleotides across the genotypes and brown colored fonts indicate the SNPs. The names of the rice genotypes are indicated to the left of each DNA sequence and the number of bases at each section is indicated at the top right corner. The two brown colored underlines at the corners indicate the position of the *K46* primer pair.

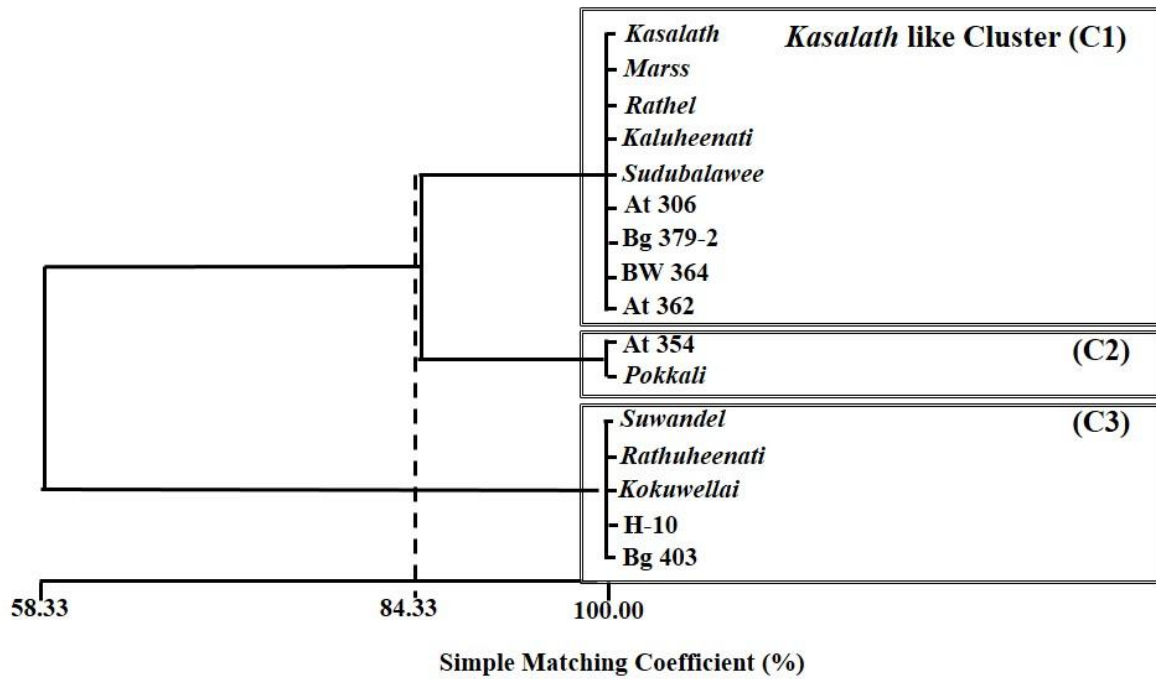


Figure 3. The dendrogram constructed based on the haplotypic variation in *K46* locus of 15 rice cultivars. Three clusters were obtained and named as C1, C2 and C3. The C1 is also named as *Kasalath*-like cluster as it contains the standard PD tolerance landrace *Kasalath*. The set of 15 cultivars in which the *K46* locus is absent was considered as the C4 cluster and is not depicted in the dendrogram.

The absence of *K46* locus in the cultivars strongly imply the fact that they also lack the INDEL region of *Pup1* as in *Nipponbare* yet possess unrevealed molecular mechanisms to confer the tolerance for PD tolerance. However the possibility of the presence of paralogous *K46* like loci in elsewhere of the genome of these cultivars cannot be simply ruled out (Thiel *et al.*, 2009). Therefore further studies are required to locate the *K46* locus of the genomes of these cultivars using techniques such as fluorescent in-situ hybridization (FISH) and linkage mapping. The utilization of FISH and linkage mapping to genomically locate the target loci is common in genetics (Rastogi *et al.*, 2013; Wissuwa *et al.*, 1998, Wissuwa, 2003). Interestingly there are two PD sensitive rice varieties Bg 357 and Bg 300 which do not have the *K46* locus and show the sensitivity to PD (Aluwihare *et al.*, 2015). The PD sensitive rice cultivar At 306 contains the *Kasalath*-like haplotype for *K46* locus indicating that the mere presence of *Kasalath*-like haplotype is not guaranteeing the PD tolerance by default. The presence of two unique *K46* haplotypes in the studied set of rice germplasm reveals the molecular diversity within *K46* locus may be related to the overall degree of PD tolerance. The association analysis between the class of haplotype and the categories of the traits is a routine diagnostic procedure in genomic studies to find the marker trait associations (Bush and Moore, 2012). The chi

square value, the Cramer's V coefficient and the associated probabilities indicated the strength and the significance of the associations (Sokal and Rohlf, 1987). In the present study it is clearly shown that *K46* haplotypes are not significantly associated with the score of PD tolerance ($p > 0.05$) (Table 1). Therefore it is safe to conclude that neither the sheer presence of *K46* locus in a rice cultivar nor the underlying haplotypic variation provide the necessary basis for marker-assisted selection. However chi-square association analysis of this nature often suffers the inherent weakness of the smaller sample size and statistical demand to have more stringent chi square thresholds (McDonald, 2014). However, in practical genetics and breeding research designs such kind of bigger sample sizes cannot be achieved as certain haplotype vs. trait category combinations are naturally rare and not available in abundance for studying. Although these complexities are present in many polygenic eukaryotic traits such as PD tolerance in rice, the value of having DNA markers as selections tools in breeding cannot be undermined (Xu and Crouch, 2008). Therefore marker validation is very much required and Xu and Crouch, (2008) clearly explained what steps are required in MAB to go from publications to real practice. It would be compulsory to molecularly characterize the entire *Pup1* region of a much larger panel of rice cultivars that are important to the country specific breeding programs. More in-depth

analysis for QTLs with genome scans using novel state of the art SNP (Chen *et al.*, 2014; Zhao *et al.*, 2011) and genotyping by sequencing (GBS) (Poland and Rife, 2012; Arbelaez *et al.*, 2015) platforms would be necessary to dig for any potential QTLs other than the *Pup1*. Although it is very expensive, such kind of and large scale research efforts are required to accurately establish the MAB platforms to produce PD tolerant rice varieties to conduct profitable rice farming with less input in the future.

CONCLUSION

The *K46* marker locus within the *Pup1* QTL is present only in the 15 rice Sri Lankan cultivars studied. There are four *K46* haplotypes within the studied set of cultivars namely *Kasalath* like haplotype, two unique haplotypes and the 'null' haplotype (i.e. the INDEL region is missing). However these haplotypes are not significantly associated with the degree of PD tolerance suggesting the presence of hitherto unknown mechanisms of PD tolerance in the local germplasm.

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Appendix 1. Rice cultivars characterized for the DNA sequence polymorphism in *K46* locus and their PD tolerance levels

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

^a The score of PD tolerance as indicated in Aluwihare *et al.*, (2015)