Ceylon Journal of Science 45(2) 2016: 87-101
DOI: http://doi.org/10.4038/cjs.v45i2.7392

RESEARCH ARTICLE

Screening of segregating F_2 progenies and validation of DNA markers through bulk segregant analysis for phosphorous deficiency tolerance in rice

Y.C. Aluwihare[1,2], M.D.M. Chamikara[1,5], N.N.H. Karannagoda[1], D.R.R.P. Dissanayake[1], R.A.G.B. Ranawaka[1], M.I. Tennakoon[1], D.N. Sirisena[3], W.L.G. Samarasinghe[3], C.K. Weebadde[4] and S.D.S.S. Sooriyapathirana[1*]

[1]*Department of Molecular Biology and Biotechnology, Faculty of Science, University of Peradeniya, Peradeniya, 20400, Sri Lanka.*
[2]*Postgraduate Institute of Science, University of Peradeniya, Peradeniya, 20400, Sri Lanka.*
[3]*Rice Research and Development Institute, Bathalagoda, Ibbagamuwa, 60500, Sri Lank.a*
[4]*Departments of Plant, Soil and Microbial Sciences and Horticulture, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI, 48824, USA.*
[5]*Current Address: Structural Biology and Biophysics Laboratory, Research School of Chemistry, Australian National University, Canberra ACT 0200, Australia.*

Received: 20 April 2016; Accepted: 20 August 2016

Abstract: Phosphorous deficiency (PD) tolerance is a polygenic trait. The underlying genetics of PD tolerance trait is important to provide the basis for detecting Quantitative Trait Loci (QTLs) and validating markers that could be used in Marker Assisted Breeding (MAB) in rice. The PD tolerance of Sri Lankan rice germplasm has been characterized. However, no attempts were taken to develop and validate the DNA markers for the breeding purposes and to understand the genetic basis of the traits. The present research project was conducted to assess the PD related traits and to validate internationally published DNA markers that are linked to PD tolerance using Sri Lankan rice cultivars. A total of 84 crosses were made and advanced to F_2 and higher generations. Out of these crosses, an important subset of three crosses was selected based on the overall PD tolerance and sensitivity, importance as mega production varieties and pedigree connections between the cultivars. The plant height, number of tillers, shoot dry weight, leaf width, flag leaf width and the color metrics L*, a*, b*, hue (h*) and chroma (C*) were measured from 200 individuals each from the three populations grown under P deficient (P_o) soil conditions. Except color traits, other traits were normally distributed and exhibited higher broad sensitivity. The color metrics indicate the presence of possible epistatic interactions between the major underlying loci. From each population, two extreme bulks were selected from the highest and lowest ends of shoot dry weight (SDW) for bulk segregant analyses (BSA) to validate the DNA markers for PD tolerance. It was observed that, DNA marker *K46-K1* can be used for MAB of rice for PD tolerance. The genetic information generated in the present study can also be used for larger scale genomic studies such as SNPs, GBS and GWAS mapping.

Keywords: Phosphorous Deficiency Tolerance, Marker Assisted Breeding, Rice Landraces, *K46-K1* marker, Shoot Dry Weight.

INTRODUCTION

Lack of required levels of phosphorous (P) in the soil is a major drawback for profitable rice farming (Marschner and Marschner, 2012; Nielsen *et al*., 2001). Application of P fertilizer is expensive and can cause major setbacks such as environmental pollution and health hazards (Bennett *et al*., 2001; Cordell *et al*., 2009; Reddy *et al*., 1999). The production of P deficiency (PD) tolerant rice varieties through marker assisted breeding (MAB) is regarded as the most logical and cost effective solution to answer this problem (Collard *et al*., 2005). Although MAB for PD tolerance in rice is widely studied in other countries (Ni *et al*., 1998; Wissuwa *et al*., 1998; Wissuwa, 2005), it has not gained too much attention in Sri Lanka. Recently, a set of rice landraces and varieties were screened for PD tolerance in Sri Lanka (Aluwihare *et al*., 2016). However, there are no attempts being made to validate the DNA markers that are linked to PD

**Corresponding Author's Email*: sunethss09@gmail.com

tolerance for Sri Lankan rice germplasm. When employing DNA markers in MAB, it requires the careful validation through genetic analysis (Xu and Crouch, 2008). The major QTL conferring PD tolerance in rice, *Pup1*, has been identified (Wissuwa and Ae, 2001a; Wissuwa *et al*., 2002), fine mapped, bioinformatically and genomically characterized (Gamuyao *et al*., 2012; Heuer *et al*., 2009) and *Pup1* linked DNA markers were developed (Chin *et al*., 2010; Chin *et al*., 2011). In order to validate these markers for a country-specific rice germplasm, bulk segregant analysis (BSA), a short cut procedure to validate DNA markers through phenotypically distinct sets of individuals in segregating populations can be employed (Venuprasad *et al*., 2009). In addition, the underlying DNA sequence variations of the marker loci could be used to detect the association between DNA polymorphisms with the traits such as PD tolerance in rice. Therefore, the present study was conducted to validate the *Pup1* linked DNA markers using BSA of F_2 populations segregating for PD tolerance.

MATERIALS AND METHODS

Plant material

A total of 12 landraces and 18 rice varieties were screened for PD soil conditions and subjected to three-tier indexation of 3: tolerant; 2: moderately tolerant and 1: sensitive (Aluwihare *et al*., 2016). Approximately 84 crosses were made between tolerant and moderately tolerant / sensitive rice genotypes using clipping and hot water dipping methods (Tong and Yoshida, 2008). A total of three crosses namely H-4 × Bg 357, *Murungakayan* × Bg 357 and *Marss* × Bg 357 were selected for the present study based on the degree of tolerance and importance to breeding. The collected F_1 seeds were planted at Rice Research and Development Institute (RRDI), Bathalagoda, Sri Lanka and F_1 plants were prudently examined to remove any off types occurred due to selfing. The F_2 seeds were collected from F_1 plants and 200 seeds (i.e. individuals) from each progeny were planted in a greenhouse at the University of Peradeniya, Sri Lanka. Ultisol soil which was collected from a field at RRDI was used as the growth medium where the field has been maintained without addition of any fertilizer for the last 40 years. The ulitsol soil was characterized for very low concentration of P (1 mg of P in 1 Kg of soil) and other nutrients (Kumaragamage and Indraratne, 2011; Sirisena and Wanninayake 2014). The standard fertilizer dressings (without P) and other management practices were applied based on the guidelines of Department of Agriculture (DOA) (Department of Agriculture, 2006), Sri Lanka.

Collection of trait data related to PD tolerance

Plant height (PlH), number of tillers (NT), shoot dry weight (SDW), leaf width (LW) and width of the middle region of the flag leaf (FLW) were measured at the early flowering stage immediately after the onset of first panicle of the plant.

The color variation occurred in leaves due to PD conditions were captured in four replicates using the color metrics L*, a*, b*, chroma (C*) and hue angle (h*) employing a spectrophotometer (CR-10, Konika Minolta, Tokyo, Japan). The L*, a* and b* indicate black / white, green / red and blue / yellow respectively. The C* and h*, are calculated based on a* and b*, indicating the overall color and sharpness of the color respectively (Figure1).

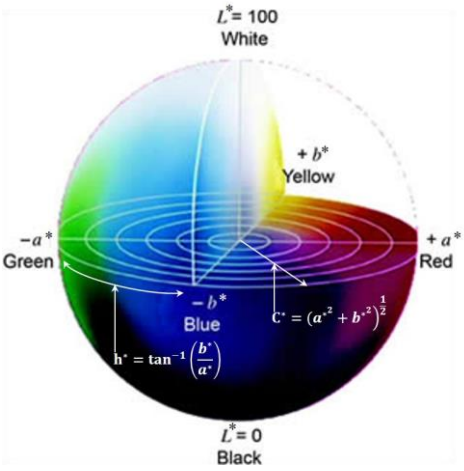

Figure 1: The universal color metric system employed in measuring leaf color variation caused by P starvation in rice.

(modified from the images available at www.colorcodehex.com and www.rodsmith.org.uk).

Table 1: The *Pup1* linked DNA markers selected for bulk segregant analysis based on the parental polymorphisms.

DNA Marker	Ta (°C)	H-4 × Bg 357	*Murungakayan* × Bg 357	*Marss* × Bg 357
RM28073	55	✓	✓	✓
K20 K/NF, NR, KR (Multiplex)	57	✓	✓	✓
K29-K	55	✓	✓	✓
K29-N	55	-	-	✓
K46	57	-	-	✓
K46-1	57	-	-	✓
K46-K1	57	-	-	✓
K46-K2	57	✓	✓	✓
K46-CG1	57	✓	-	✓
K48	57	✓	✓	-
K52	57	✓	-	-
K59	57	✓	-	✓

Tick marks indicate that two parents have polymorphic loci for the particulate DNA marker

Table 2: The F2 individuals selected for Bulk Segregated Analysis (BSA) based on the highest and the lowest shoot dry weight (SDW) values from three populations

Bulk	No.	IDs of the selected individuals from F_2 populations		
		H-4 × Bg 357	***Murungakayan* × Bg 357**	***Marss* × Bg 357**
Lowest SDW	1	H-4 × Bg 357_028	*Murungakayan* × Bg 357_111	*Marss* × Bg 357_199
	2	H-4 × Bg 357_133	*Murungakayan* × Bg 357_095	*Marss* × Bg 357_050
	3	H-4 × Bg 357_142	*Murungakayan* × Bg 357_064	*Marss* × Bg 357_020
	4	H-4 × Bg 357_129	*Murungakayan* × Bg 357_140	*Marss* × Bg 357_161
	5	H-4 × Bg 357_018	*Murungakayan* × Bg 357_027	*Marss* × Bg 357_160
	6	H-4 × Bg 357_059	*Murungakayan* × Bg 357_108	*Marss* × Bg 357_074
	7	H-4 × Bg 357_049	*Murungakayan* × Bg 357_092	*Marss* × Bg 357_033
	8	H-4 × Bg 357_197	*Murungakayan* × Bg 357_021	*Marss* × Bg 357_063
Highest SDW	1	H-4 × Bg 357_144	*Murungakayan* × Bg 357_122	*Marss* × Bg 357_118
	2	H-4 × Bg 357_173	*Murungakayan* × Bg 357_121	*Marss* × Bg 357_004
	3	H-4 × Bg 357_057	*Murungakayan* × Bg 357_100	*Marss* × Bg 357_179
	4	H-4 × Bg 357_126	*Murungakayan* × Bg 357_174	*Marss* × Bg 357_045
	5	H-4 × Bg 357_109	*Murungakayan* × Bg 357_173	*Marss* × Bg 357_189
	6	H-4 × Bg 357_158	*Murungakayan* × Bg 357_182	*Marss* × Bg 357_103
	7	H-4 × Bg 357_120	*Murungakayan* × Bg 357_045	*Marss* × Bg 357_073
	8	H-4 × Bg 357_121	*Murungakayan* × Bg 357_181	*Marss* × Bg 357_146

DNA marker polymorphism detected through bulk segregant analysis

A total of 12 *Pup1* specific DNA markers were employed in validating the polymorphism through BSA of the three F_2populations namely H-4 × Bg 357, *Murungakayan* × Bg 357 and *Marss* × Bg 357. The markers were detected based on the polymorphic bands present among

the parental genotypes explained in Table 1. A total of eight individuals, each having highest and the lowest SDW, were selected from each F_2 population for the BSA (Table 2). The immature leaf samples were used to extract DNA using Dneasy® plant mini kit (Qiagen, Solna, Sweden). The *trnH-psbA*, a standard and universal plant DNA barcoding primer pair (Hollingsworth *et al*., 2011), was used to validate the quality and rightness of DNA for PCR amplification. The DNA from each eight individuals were bulked and used as the template DNA in PCR. For each F_2 population, DNA of two parents, DNA from two bulks (highest and lowest SDW groups) and DNA from the individuals from each bulk (i.e. a total of 16 individuals) were used for PCR using the DNA markers given in Table 1.The PCR conditions were provided using a Thermal Cycler (Takara, Japan) as follows; initial denaturation at 94 °C for 5 min, then 35 cycles of 94 °C for 30 sec, primer annealing temperature (Ta) (Table 1) for 90 sec, and 72 °C for 2 min, final extension at 72 °C for 10 min.The amplified PCR products were size separated using 1.5 % ethidium bromide stained agarose gel electrophoresis.

Data analysis

Statistical analysis of PD tolerance data

All the tested parameters were used to calculate the Pearson's Correlation Coefficients (PCC) using the statistical package Minitab 16 (Minitab Inc., USA). Kolmogorov-Smirnov (KS) Coefficient, skewness and kurtosis were calculated to test the normality of trait distributions. The data distributions were plotted as histograms and parental values were marked to understand the data range and the presence of transgressive segregants.

Genetic analysis for heritability, heterosis and goodness of fit for epistasis

The Broad Sense Heritability (BSH), Mid Parental Heteros is (MPH), Better Parental Heterosis (BPH) and Worse Parental Hetero sis (WPH) were calculated according tothe formula given in Falconer and Mackay, (1996) and Mukamuhirwa *et al*.,(2015). The color metrics, L*, a*, b*, C* and h*, were subjected to goodness of fit analyses with respect to the dihybrid ratios 9:3:4, 9:6:1, 10:3:3 and 12:3:1 using the Statistical Software *SOCR* version 1.1.

RESULTS

Correlation among tested parameters

The PlH was not significantly correlated with NT. However, PlH and NT were significantly correlated with SDW separately (PCC of 18 % - 46 %). In H-4 × Bg 357 population, NT was significantly correlated with LW but in other two populations, LW was correlated with PlH. In H-4 × Bg 357 and *Marss*× Bg 357 populations, FLW was significantly correlated with PlH and LW. The color parameters L* and a* were strongly and negatively correlated with a* (PCC of -60 % to -81 %). The color parameter b* was also correlated with L* and a* separately. The integrative color metrics, C* and h*, were significantly correlated with L*, a* and b* except in H-4 × Bg 357 and *Marss* × Bg 357 populations, where b* was not correlated with C* and h* (Table 3) ($P<0.05$).

The nature of the trait distribution

The parameters PlH, NT, LW and FLW were normally distributed in all three populations. The SDW was not normally distributed in *Murungakayan* × Bg 357 and *Marss* × Bg 357. All five color parameters were not significantly and normally distributed in all three populations. The nature of the distribution and presence of transgressive segregants with respect to the parental trait values are shown in Figure 2, 3, 4 and 5.

Heritability and heterosis

The BSH was higher for all the traits in all three populations ranging from 34 % to 98 %. Heterosis(H) was present in all traits. The detailed,H and BSH values are given in Table 4.

Epistatic nature of the inheritance in color metrics

A goodness of fit analysis for the epistatic ratios revealed that the color metrics b* and h* in H-4 × Bg 357 population, a*, b* and h* in *Murungakayan* × Bg 357 population and L* and h* in *Marss* × Bg 357 population were fitting into the epistatic ratio of 12:3:1. The color metrics b* and h* in *Murungakayan* × Bg 357, b* and a* in *Marss* × Bg 357 were fitting into 10:3:3 ratio. The 9:6:1 ratio was not observed in any of the populations and the 9:3:4 ratio was matched with the color metric b* in *Marss*× Bg 357 ($P<0.05$) (Table 5).

Table 3: The Pearson's Correlation Coefficients (PCC) among the traits measured for F_2 progenies grown under PD conditions

Progeny		NT	SDW	LW	FLW	L*	a*	b*	C*	h*
H-4 ×Bg 357	**PlH**	-0.04	0.18**	0.07	-0.12**	0.11	-0.11	0.08	0.09	-0.09
	NT		0.18**	0.20**	-0.01	0.09	-0.07	-0.03	0.07	0.03
	SDW			0.05	0.01	-0.04	0.05	0.01	-0.05	-0.01
	LW				0.43***	0.07	0.02	-0.02	-0.01	0.02
	FLW					-0.08	0.04	0.05	-0.05	-0.04
	L*						-0.81***	0.15*	0.81***	-0.26***
	a*							-0.16*	-0.99***	0.30***
	b*								0.02	-0.99***
	C*									-0.17
Murungakayan× Bg 357	**PlH**	0.04	0.46***	0.18**	0.04	-0.06	0.03	0.08	-0.04	-0.07
	NT		0.42***	0.12	-0.12	-0.17	0.08	0.01	-0.09	0.001
	SDW			0.16*	0.05	-0.08	0.04	0.01	-0.05	-0.004
	LW				0.55	-0.006	-0.04	-0.005	0.04	-0.003
	FLW					0.07	-0.15	0.02	0.15	-0.04
	L*						-0.81***	0.33***	0.81***	-0.44***
	a*							0.33***	-0.99***	0.47***
	b*								0.24***	-0.98***
	C*									-0.39***
Marss×Bg 357	**PlH**	0.06	0.40***	0.23***	0.19**	-0.12	-0.04	-0.02	0.05	0.02
	NT		0.30***	0.11	-0.04	0.06	-0.12	0.09	0.11	-0.11
	SDW			0.11	0.15	-0.11	0.08	0.04	-0.09	-0.02
	LW				0.65***	-0.11	-0.03	0.09	0.13	-0.10
	FLW					-0.07	0.06	-0.03	-0.06	0.03
	L*						-0.6***	0.23***	0.60***	-0.29***
	a*							-0.26***	-0.99***	0.41***
	b*								0.13	-0.98***
	C*									-0.29***

* P<0.05, **P<0.01, ***P<0.001

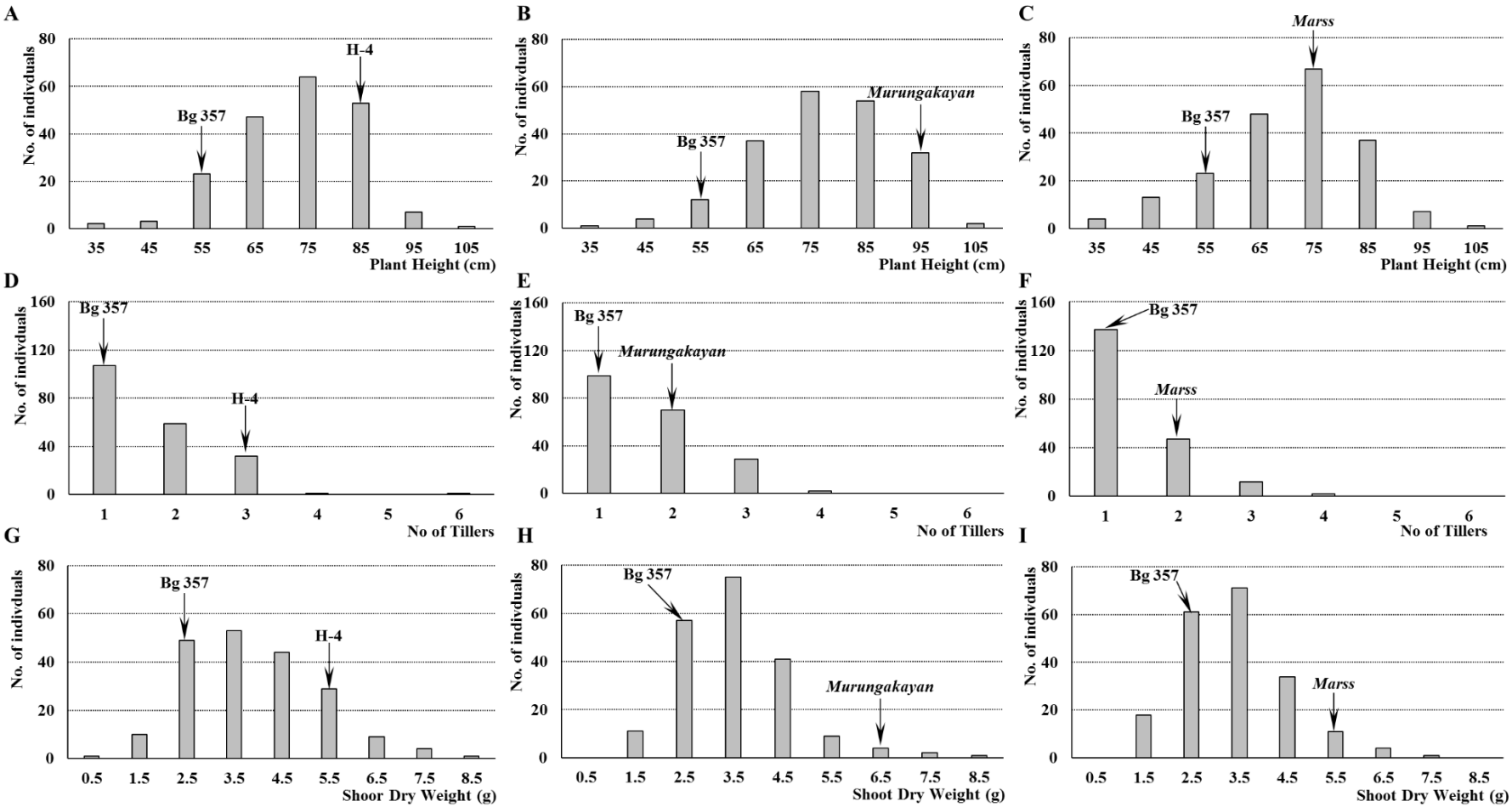

Figure 2: Frequency distribution of the F_2 progenies for plant height (PlH), number of tillers (NT) and shoot dry weight (SDW). The graphs A, B and C represent PlH; E, F and G represent NT; I, J and K represent SDW. In vertical direction the graphs A, D and G represent the progeny H-4 × Bg 357; B, E and H represent *Murungakayan*× Bg 357; C, F and I represent *Marss* × Bg 357. The positions of the parental trait values are shown with the arrows. The class midpoints of each phenotypic trait are shown in the X axis.

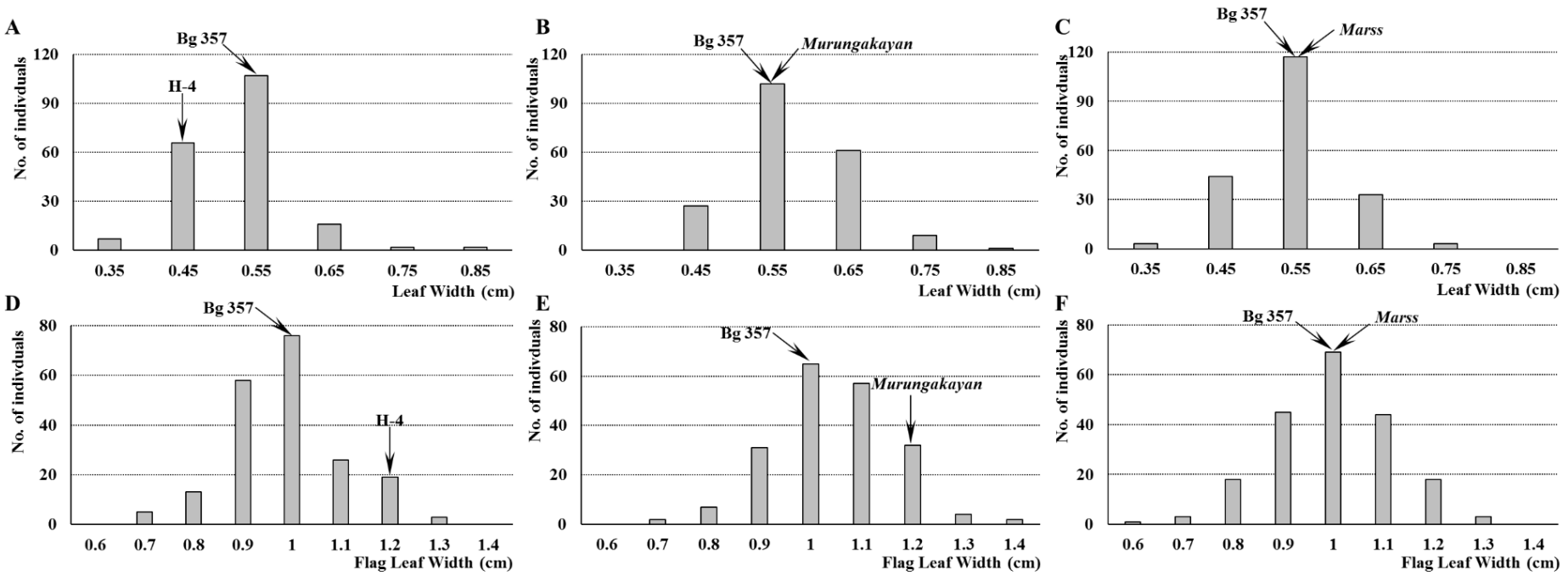

Figure 3: Frequency distribution of the F_2 progenies for leaf width (LW) and flag leaf width (FLW). The graphs A, B and C represent LW; D, E and F represent FLW. In vertical direction the graphs A and D represent the progeny H-4 × Bg 357; B and E represent *Murungakayan*× Bg 357; C and F represent *Marss* × Bg 357. The positions of the parental trait values are shown with the arrows. The class midpoints of each phenotypic trait are shown in the X axis.

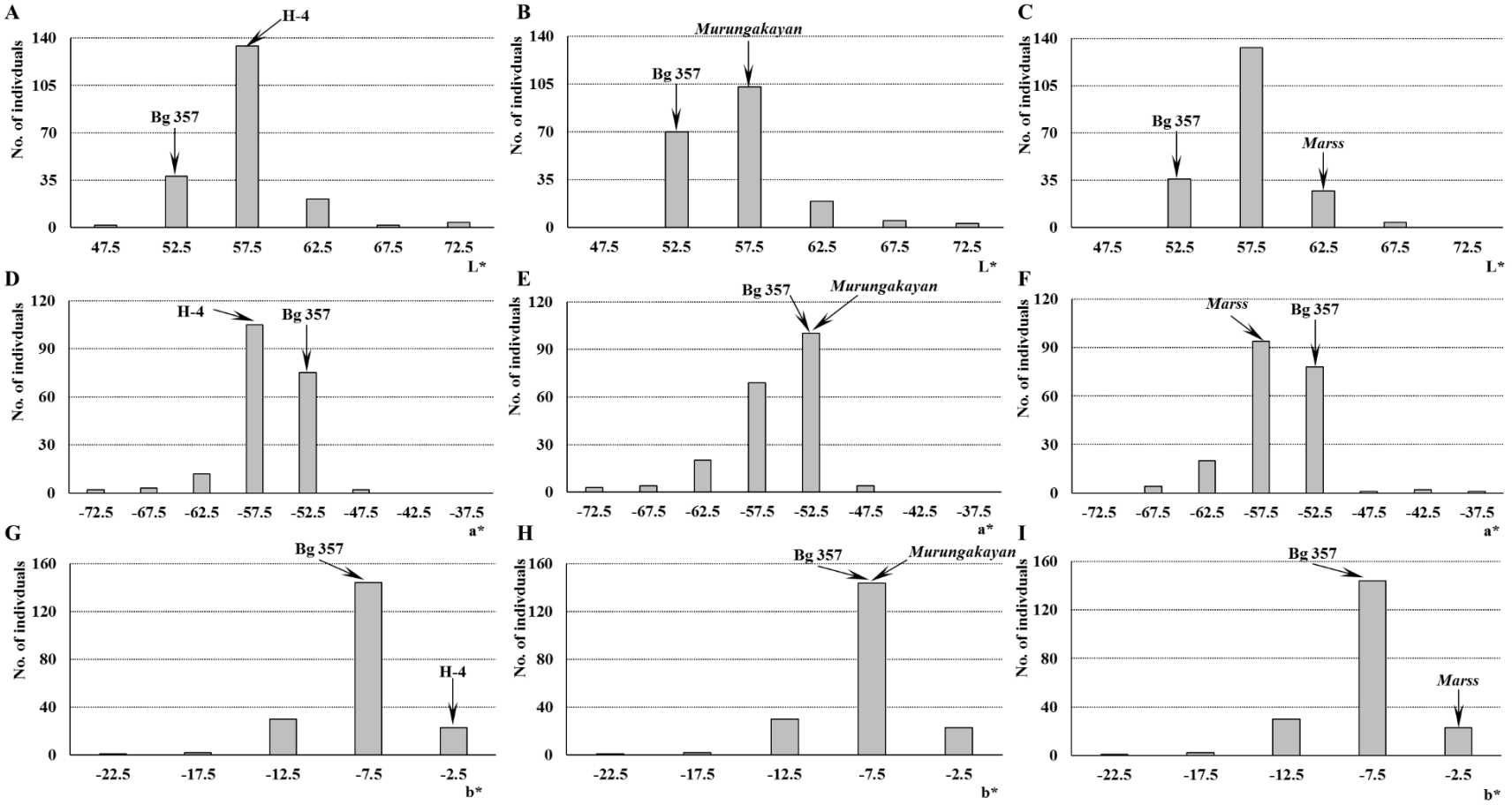

Figure 4: Frequency distribution of the F_2 progenies for L*, a* and b*. The graphs A, B and C represent L*; D, E and F represent a*; G, H and I represent b*. In vertical direction the graphs A, D and G represent the progeny H-4 × Bg 357; B, E and H represent *Murungakayan*× Bg 357; C, F and I represent *Marss* × Bg 357. The positions of the parental trait values are shown with the arrows. The class midpoints of each phenotypic trait are shown in the X axis.

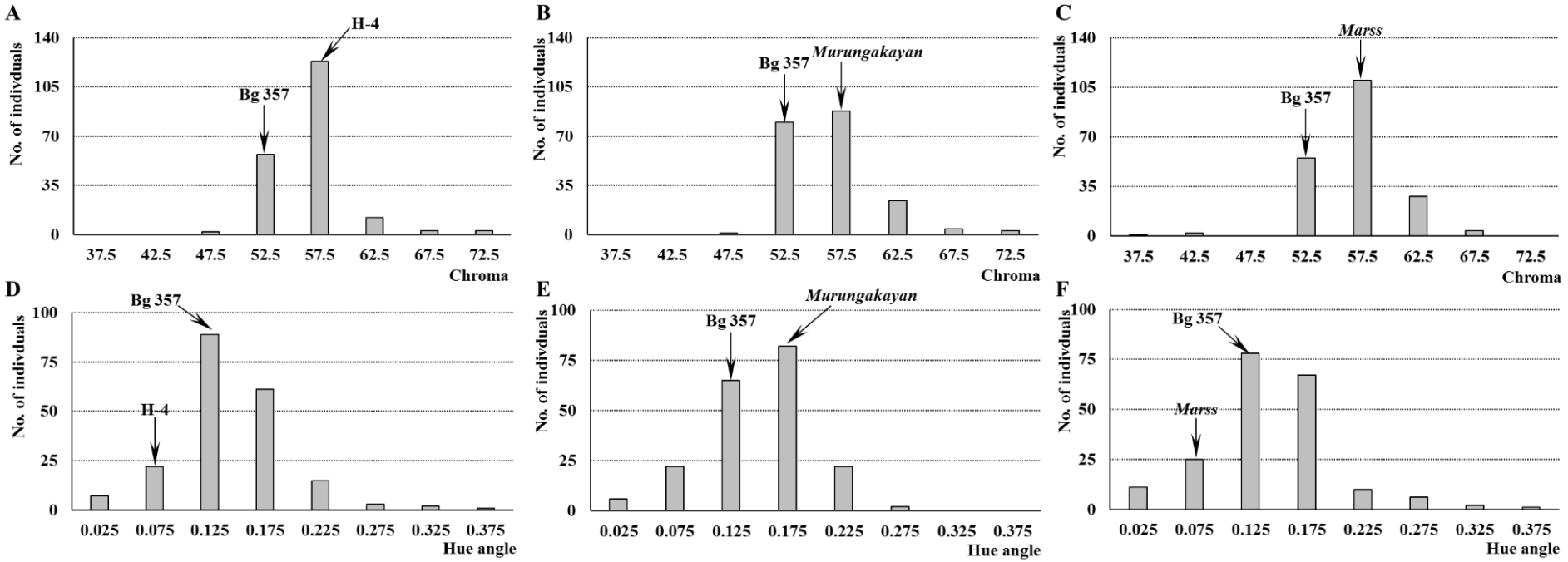

Figure 5: Frequency distribution of the F_2 progenies for chroma (C*) and hue angle (h*). The graphs A, B and C represent C*; D, E and F represent h*. In vertical direction the graphs A and D represent the progeny H-4 × Bg 357; B and E represent *Murungakayan*× Bg 357; C and F represent *Marss* × Bg 357. The positions of the parental trait values are shown with the arrows. The class midpoints of each phenotypic trait are shown in the X axis.

Table 4: The normality, heritability and heterosis estimates of the traits measured under PD conditions of the F_2 populations

Cross	Trait	Mean	Normality as indicated by the KS coefficient[#]	Skewness	Kurtosis	Broad Sense Heritability (%)	Mid Parental Heterosis (%)	Better Parental Heterosis (%)	Worse Parental Heterosis (%)
H-4 × Bg 357	Plant height (cm)	72.47	0.05	-0.31	0.07	94.71	7.07	-10.94	34.20
	Tiller number	1.66	0.06	1.32	2.70	73.42	-8.41	-33.60	47.56
	Shoot dry weight (g)	3.92	0.06	0.58	0.35	93.65	-2.74	-34.58	89.49
	Leaf width (cm)	0.52	0.06	1.05	4.22	58.72	-0.23	-9.99	11.90
	Flag leaf width (cm)	0.99	0.03	0.22	0.22	86.64	23.75	-3.41	72.17
	L*	57.04	0.11**	1.47	5.51	69.49	3.96	-2.78	11.72
	a*	-55.91	0.13**	-1.67	5.36	71.75	2.69	-2.51	8.47
	b*	-7.84	0.09**	-1.13	4.67	97.53	51.08	80.91	29.69
	Chroma	56.53	0.13**	1.67	5.20	70.58	3.33	-1.71	8.92
	Hue angle	0.14	0.07*	0.78	2.71	97.69	45.67	85.81	19.80
Murungakayan × Bg 357	Plant height (cm)	76.66	0.05	-0.47	0.18	94.47	5.74	-15.76	41.96
	Tiller number	1.67	0.06	0.77	-0.37	74.01	-1.04	-25.78	48.44
	Shoot dry weight (g)	3.53	0.08**	1.10	2.31	85.47	-13.17	-41.77	70.63
	Leaf width (cm)	0.57	0.05	0.42	0.50	58.31	2.47	-0.87	6.05
	Flag leaf width (cm)	1.05	0.01	-0.00	0.26	76.00	-0.08	-0.16	0.02
	L*	56.74	0.14**	1.71	3.97	84.31	6.20	1.70	2.18
	a*	-55.62	0.23**	-1.55	3.20	82.44	4.67	1.60	7.94
	b*	-8.02	0.12**	0.27	0.56	97.82	9.90	-4.61	32.67
	Chroma	56.26	0.15**	1.56	3.22	81.63	4.88	1.57	8.40
	Hue angle	0.14	0.06	-0.24	0.32	97.01	3.19	-8.85	-98.07
Marss × Bg 357	Plant height (cm)	69.54	0.06	-1.08	3.17	95.99	7.50	-7.74	28.78
	Tiller number	1.41	0.07*	1.74	2.73	62.90	-22.21	-43.60	25.33
	Shoot dry weight (g)	3.35	0.10**	0.57	0.71	91.11	-7.19	-34.95	61.93
	Leaf width (cm)	0.53	0.06	0.27	0.61	59.93	-3.64	-7.83	0.95
	Flag leaf width (cm)	1.00	0.01	-0.12	0.11	90.17	-1.84	-2.44	-1.23
	L*	57.19	0.09**	0.90	1.44	34.00	2.16	-6.10	12.01
	a*	-55.94	0.11**	0.45	5.47	60.01	0.66	-6.16	8.56
	b*	-7.07	0.08**	1.17	6.74	98.06	53.07	91.37	27.54
	Chroma	56.56	0.12**	-0.56	5.46	57.87	1.32	-5.34	8.98
	Hue angle	0.14	0.06*	0.51	1.77	98.03	51.81	107.30	19.76

[#]Significant levels for Kolmogorov–Smirnov (KS) Coefficient: * P <0.05, ** P <0.01
(Significant KS indicates the deviation)

The maker validation through bulk segregant analysis

The SDW based BSA did not provide tolerant or sensitive PD class specific marker alleles exclusively(Figure 6 and Table 6). However, *K46-K1* marker allele was more specific to tolerant group indicating its applicability in MAB.

Table 5: Goodness of fit analyses for color metric data measured under PD conditions in F_2populations

		Calculated Chi Square Values of Goodness of Fit for potential expected dihybrid ratios in F_2 progenies			
Cross	**Color metric**	**9:3:4**	**9:6:1**	**10:3:3**	**12:3:1**
H-4 × Bg 357	L*	56.72***	36.89***	25.53***	4.83
	a*	98.27***	94.64***	69.36***	26.15***
	b*	23.94***	41.16***	9.04*	9.60**
	Chroma	98.19***	113.65***	70.82***	40.97***
	Hue angle	32.59***	45.44***	15.17***	6.45*
Murungakayan × Bg 352	L*	64.25***	2.76	51.45***	35.01***
	a*	73.64***	64.88***	48.36***	11.13**
	b*	21.98***	30.74***	8.04*	8.09*
	Chroma	74.91***	119.52***	54.26***	55.54***
	Hue angle	26.63***	50.86***	11.19**	13.11**
Marss × Bg 357	L*	62.44***	46.12***	39.79***	6.51***
	a*	78.65***	71.93***	52.46***	13.87***
	b*	10.91**	56.94***	1.78	32.45***
	Chroma	62.72***	124.04***	45.35***	63.66***
	Hue angle	28.67***	33.05***	12.39**	3.61

*P <0.05 (Expected χ^2 value: 5.99), **P <0.01 (Expected χ^2 value: 9.21), ***P <0.001 (Expected χ^2 value: 13.82)

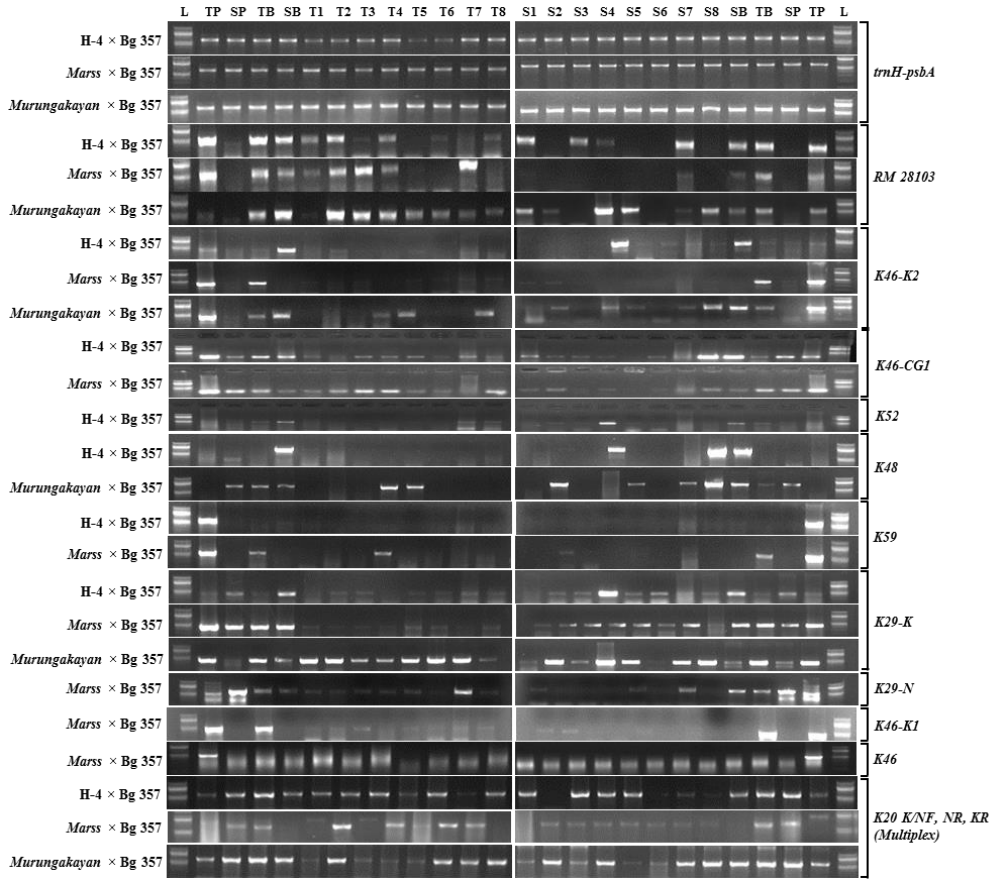

Figure 6: The composite gel image for 13 *Pup1* linked DNA markers subjected to bulk segregant analysis using the selected bulks based on higher and lower shoot dry weights in three F_2 populations. The F_2 populations were screened under PD conditions. Marker names are indicated at the right and population names are indicated at the left. The top row represents the sample labels. TP: PD tolerant parent, SP: PD sensitive parent; TB: PD tolerant bulk (consist the mixture of DNA from T1 to T8); SB: PD sensitive bulk (consist the mixture of DNA from S1 to S8). T: Tolerant; S: Sensitive. The DNA barcoding primer pair *trnH-psbA* was used to confirm the quality of DNA for PCR.

Table 6: The *Pup1* linked DNA marker polymorphisms detected in three F_2 populations based on the BSA

DNA marker allele	F_2 population	TP	SP	TB	SB	Percentage individuals in each class having the allele	
						Tolerant	Sensitive
RM28073	H-4 × Bg 357	1	0	1	1	75	50
	Marss × Bg 357	1	0	1	1	63	13
	Murungakayan × Bg 357	1	0	1	1	100	75
K29-K	H-4 × Bg 357	1	1	1	1	75	88
	Marss × Bg 357	1	1	1	1	100	100
	Murungakayan × Bg 357	1	1	1	1	100	88
K29-N	*Marss* × Bg 357	1	1	1	1	88	38
K46	*Marss* × Bg 357	1	0	1	1	38	100
K46-K1	*Marss* × Bg 357	1	0	1	0	100	25
K46-K2	H-4 × Bg 357	1	0	0	1	25	50
	Marss × Bg 357	1	0	1	0	50	25
	Murungakayan × Bg 357	1	0	1	1	38	63
K46-CG1	H-4 × Bg 357	1	1	1	1	100	100
	Murungakayan × Bg 357	1	1	1	1	100	75
K48	H-4 × Bg 357	0	1	0	1	00	38
	Murungakayan × Bg 357	0	1	1	1	25	50
K52	H-4 × Bg 357	1	1	1	1	25	13
K59	H-4 × Bg 357	1	0	0	0	00	00
	Marss × Bg 357	1	0	1	0	13	13
K20 K/NF, NR, KR (Multiplex)	H-4 × Bg 357	1	1	1	1	100	63
	Marss × Bg 357	1	1	1	0	75	75
	Murungakayan× Bg 357	1	1	1	1	100	100

DISCUSSION

The dissection of the genetic basis for PD tolerance is not easy as it is a polygenic trait. However, the heritability associated with the traits of PD tolerance is found to be higher, enabling the possible detection of underlying QTLs (Majumder *et al*., 1989). Identification of the nature of inheritance in traits such as PD tolerance can be done by using the phenotypic variations in segregating populations. In the present study, more than 80 F_1 crosses were made between the PD tolerant and PD sensitive rice cultivars reported in Aluwihare *et al*., (2016). These crosses are currently being advanced towards ~F_{10} with selections to produce Recombinant Inbred Lines (RILs) in the breeding programs conducted by RRDI, Sri Lanka. For the present study, three crosses namely H-4× Bg 357, *Murungakayan* × Bg 357 and *Marss* × Bg 357 were selected for the analysis. The selection of these crosses was mainly based on few factors. To understand the genetics of PD tolerance logically, one parent was kept as a common parent for all three crosses so that meaningful comparisons can be made across the rice cultivars. The improved rice variety Bg 357 was preferred as it is one of the most popular mega rice varieties in Sri Lanka. Parent H-4 was selected because it is the highest PD tolerant rice variety according to Aluwihare *et al*., (2016). The landraces *Murungakayan* and *Marss* were preferred because they are PD tolerant (Aluwihare *et al*., 2016) but more importantly, the parents of H-4. The selection of multiple segregating populations with shared

parents is a common practice in many genetic and breeding programs (Brim, 1966; Brown and Caligari, 2011; Kharkwal and Sharma, 2002; Suneson, 1956) and provide detailed comparisons plus the basis for association mapping such as GWAS (Bush and Moore, 2012), if resources are available.

The distribution of the phenotypic data, Skewness and Kurtosis parameters indicated that except the color metrics, other traits are mostly normally distributed except the SDW in two populations (Figure 2, 3, 4 and 5). The normal distribution provides the rationale for correct adoptions of mapping algorithms in future experiments. As Majumder *et al*., (1989) observed all tested parameters exhibit very high BSH indicating PD has a considerable proportion of genetic variation in all three populations.

The H estimates are highly variable for the traits indicating the complexity of the inheritance of the PD tolerance and it is quite premature to conclude that there is a significant dominance effect for the PD tolerance in three populations. However, transgressive segregants were observed in all traits in all three populations which could possibly be due to some form of H.

The color metrics L*, a*, b*, C* and h*, are important as the leaf color variation due to PD tolerance is not very distinct and quantitatively distributed so that a continuous scale is required to capture the variation. There are many gene mapping studies which have used these color metrics for mapping QTLs (Espley *et al*., 2007; Sooriyapathirana *et al*., 2010; Uematsu *et al*., 2014). All the color metrics exhibit non-normal distributions strongly, indicating the presence of major genes with intergenicepistatic interactions. As KS coefficient indicates, the distributions of the color metrics are strongly and significantly deviated from the normality. When the color metric data are categorized into three distinct groups for each population and subjected to chi-square value based goodness of fit analyses, some significant epistatic interactions were observed ($P<0.001$). The color metric L* is following 9:6:1 ratio for *Murungakayan* and Bg 357 populations. The color metric b* in *Marss*× Bg 357 population is following 10:3:3 epistatic ratio and color metric L* in H-4 × Bg 357 and color metric h* in *Marss* × Bg 357 population are following 12:3:1 interactions. The other color traits were not significant for the epistatic ratios possibly because only 200 individuals were screened for each population. However, this goodness of fit analyses provide the basis that, there could be some major QTLs conferring the leaf color change due to P starvation which includes complex effects modified by the other QTLs and the environment.

The marker validation and QTL mapping using the populations in which each is having 200 individuals are expensive and tedious to undertake (Xu and Crouch, 2008). The BSA has been introduced as the shortcut method to quickly validate the DNA markers with reduced inputs (Venuprasad *et al*., 2009). However, in the present study it was difficult to identify the distinct groups or traits as the bulks for BSA. Each trait is uniquely distributed and provided specific tail groups making an integrated approach of selecting bulk impossible. After conducting so many rounds of iterations to select bulks, SDW was considered as the single parameter to select the bulks. The selection of SDW as the bulk defining criterion would be logical as it is highly and significantly associated with the PD tolerance in rice (Wissuwa *et al*., 1998; Aluwihare *et al*., 2016; Wissuwa and Ae, 2001b). From each population, the eight individuals who got the highest SDW and the eight individuals having the lowest SDW were selected as the PD tolerant and sensitive bulks separately (Table 2). The selection of bulks from the trait distribution curves is very common in genetics (Harris, 1911) and especially in latest core genome based gene expression and SNP array analyses (Kearsey and Farquhar, 1998; Varshney *et al*., 2009). The BSA analysis revealed no tolerant or sensitive bands exclusively indicating the higher variability in *Pup1* QTL region. As it was clearly reviewed in Chin *et al*., (2011), the phenotyping for *Pup1* QTL is confusing and SDW cannot be used as the sole criterion for BSA. The higher transposon activity (Heuer *et al*., 2009) might complicate the genomic architecture that would make BSA quite cumbersome.

CONCLUSION

The screening of F_2 rice progenies H-4× Bg 357, *Murungakayan* × Bg 357 and *Marss* × Bg 357 segregating for PD tolerance indicates the complex nature of inheritance and higher heritability estimates. The color variation due to P starvation is not normally distributed and having epistatic gene interactions. The BSA using the tolerant and sensitive bulks selected

from the three populations have validated *K46-1* that can be used for MAB for PD tolerance in rice.

ACKNOWLEDGEMENTS

National Research Council (NRC), Sri Lanka Grant NRC11-087 for funding.

REFERENCES

Aluwihare, Y.C., Ishan, M., Chamikara, M.D.M., Weebadde, C.K., Sirisena, D.N., Samarasinghe, W.L.G. and Sooriyapathirana, S.D.S.S. (2016). Characterization and selection of phosphorus deficiency tolerant rice genotypes in Sri Lanka. *Rice Science* **23**: 184-195.

Bennett, E.M., Carpenter, S.R. and 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. *Bio Science* **51**: 227-234.

Brim, C.A. (1966). A modified pedigree method of selection in soybeans. *Crop Science***6**:220.

Brown, J. and Caligari, P. (2011). *An Introduction to Plant Breeding*. John Wiley and Sons. USA.

Bush, W.S. and Moore, J.H. (2012). Chapter 11: Genome-Wide Association Studies. *PLoS Computational Biology* **8**: e1002822.

Chin, J.H., Gamuyao, R., Dalid, C., Bustamam,M., Prasetiyono, J., Moeljopawiro, S., Wissuwa, M. and Heuer,S.(2011). Developing rice with high yield under phosphorus deficiency: *Pup1* sequence to application. *Plant Physiology* **156**: 1202-1216.

Chin, J.H., Lu, X.,Haefele, H.M., Gamuyao, R., Ismail, A.M., Heuer, S. and Wissuwa, M.(2010). Development and application of gene-based markers for the major rice QTL Phosphorus uptake 1. *Theoritical and Applied Genetics* **120**:1073-1086.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B. and Pang, E.C.K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. *Euphytica* **142**: 169-196.

Cordell, D., Drangert, J.O. and White, S. (2009). The story of phosphorus: global food security and food for thought. *Global EnvironmentalChange* **19**: 292-305.

Department of Agriculture, Sri Lanka (2006). *Climate of rice growing regions in Sri Lanka*. Available from: http://www.agridept.gov.lk/ (Accessed 10.08.2015).

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S. and Allan, A.C. (2007). Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *The Plant Journal* **49**: 414-427.

Falconer, D.S. and Mackay, T.F.C. (1996). *Introduction to Quantitative Genetics*, third ed. Longman. New York,

Gamuyao, R., Chin, J.H., Pariasca-Tanaka, J., Pesaresi, P., Catausan, S., Dalid, C., Slamet-Loedin, I., Tecson-Mendoza, E.M., Wissuwa, M. and Heuer, S. (2012). The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency. *Nature* **488**: 535-539.

Harris, J.A. (1911). The Distribution of Pure Line Means. *The American Naturalist* **45**: 686-700.

Heuer, S., Lu, X., Chin, J.H., Tanaka, J.P., Kanamori, H., Matsumoto, T., De Leon, T., Ulat, V.J., Ismail, A.M., Yano, M. and Wissuwa, M. (2009). Comparative sequence analyses of the major quantitative trait locus phosphorus uptake 1 (*Pup1*) reveal a complex genetic structure. *Plant Biotechnology Journal* **7**: 456-471.

Hollingsworth, P.M., Graham, S.W. and Little, D.P. (2011). Choosing and using a plant DNA barcode. *PLOS ONE* **6**: e19254 doi:10.1371/journal.pone.0019254.

Kearsey, M.J. and Farquhar, A.G.L. (1998). QTL analysis in plants; where are we now. *Heredity* **80**: 137-142.

Kharkwal, M. and Sharma, R.K. (2002). Principles and procedures of plant breeding-biotechnological and conventional approaches. *Indian Journal of Genetics and Plant Breeding* **62**: 386-387.

Kumaragamage, D. and Indraratne, S. (2011). Systemic approach to diagnosing fertility problems in soils of Sri Lanka. *Communications in soil science and plant analysis* **42**: 2699-2715.

Majumder, N., Borthakur, D. and Rakshit, S. (1989). Heterosis in rice under phosphorus stress. *Indian Journal of Genetics and Plant Breeding* **49**:231-235.

Marschner, H. and Marschner, P. (2012). *Marschner's mineral nutrition of higher plants*, 3rd ed. Elsevier, USA.

Mukamuhirwa, F., Tusiime, G. and Mukankusi, M.C. (2015). Inheritance of high iron and zinc concentration in selected bean varieties. *Euphytica* **205**: 349-360.

Ni, J., Wu, P., Senadhira, D. and Huang, N. (1998). Mapping QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoritical and Applied Genetics* **97**: 1361-1369.

Nielsen, K.L., Eshel, A. and Lynch, J.P. (2001). The effect of phosphorus availability on the carbon economy of contrasting common bean (*Phaseolus vulgaris* L.) genotypes. *Journal of Experimental Botany* **52**: 329-339.

Reddy, K.R., Kadlec, R.H., Flaig, E. and Gale, P.M. (1999). Phosphorus retention in streams and wetlands: A review. *Critical Reviews in Environmental Science and Technology* **29**: 83-146.

Sirisena,D.N. and Wanninayake, W.M.N.(2014). Identification of promising rice varieties for low fertile soils in the low country intermediate zone in Sri Lanka.*Annals of Sri Lanka Department of Agriculture* **14**: 95-105.

Sooriyapathirana, S.S., Khan, A., Sebolt, A.M., Wang, D., Bushakra, J.M., Kui Lin-Wang, Allan, A.C., Gardiner, S.E., Chagné, D. and Iezzoni, A.F.(2010). QTL analysis and candidate gene mapping for skin and flesh color in sweet cherry fruit (*Prunusavium* L.).*Tree Genetics and Genomes* **6**: 821-832.

Suneson, C.A. (1956). An evolutionary plant breeding method. *Agronomy Journal* **48**: 188-191.

Tong, L. and Yoshida, T.(2008). Can hot-water emasculation be applied to artificial hybridization of indica-type Cambodian rice? *Plant Production Science* **11**: 132-133.

Uematsu, C., Katayama, H., Makino, I., Inagaki, A., Arakawa, O. and Martin, C. (2014). Peace, a MYB like transcription factor, regulates petal pigmentation in flowering peach 'Genpei' bearing variegated and fully pigmented flowers. *Journal of Experimental Botany* **65**: 1081-1094.

Varshney, R.K., Nayak, S.N., May, G.D. and Jackson, S.A. (2009). Next-generation sequencing technologies and their implications for crop genetics and breeding. *Trends in Biotechnology* **27**: 522-530.

Venuprasad, R., Dalid, C.O., Valle, M.D., Zhao, D., Espiritu, M., Sta Cruz, M.T.,Amante, M., Kumar, A. and Atlin, G.N. (2009). Identification and characterization of large-effect quantitative trait loci for grain yield under lowland drought stress in rice using bulk-segregant analysis. *Theoritical and Applied Genetics* **120**: 177-190.

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.

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

Wissuwa, M. and Ae, N. (2001b). Genotypic variation for tolerance to phosphorus deficiency in rice and the potential for its exploitation in rice improvement. *Plant Breeding* **120**: 43-48.

Wissuwa, M., Wegner, J., Ae, N. and Yano, M. (2002). Substitution mapping of *Pup1*: a major QTL increasing phosphorus uptake of rice from a phosphorus-deficient soil. *Theoritical and Applied Genetics* **105**: 890-897.

Wissuwa, M., Yano, M. and Ae, N. (1998). Mapping of QTLs for phosphorus deficiency tolerance in rice (*Oryza sativa* L.). *Theoritical and AppliedGenetics* **8**: 777-783.

Xu, Y. and Crouch, J.H. (2008). Marker assisted selection in plant breeding: from publications to practice. *Crop Science* **48**: 391-407.