Stacking of Pup1 QTL for Low Soil Phosphorus Tolerance and Bacterial Blight Resistance Genes in the Background of APMS6B, the Maintainer Line of Rice Hybrid DRRH-3

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Research Article

Keywords: Rice, Low phosphorus tolerance, Bacterial Blight, maintainer line, APMS6B, MABB, hybrids.

Posted Date: September 30th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-883401/v1

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Acknowledgement: Authors are grateful to Indian Council of Agriculture Research (ICAR), ICAR-Indian Institute of Rice Research (ICAR-IIRR) and Govt. of India for their financial support and facilities for research.

Stacking of *Pup1* QTL for low soil phosphorus tolerance and bacterial blight resistance genes in the background of APMS6B, the maintainer line of rice hybrid DRRH-3

Abstract

Phosphorus (P) is one of the macro nutrients essential for plant growth and development. Rice (*Oryza sativa* L.) is sensitive to P starvation and its deficiency influences many key plant functions which results in crop yield penalty. Although the hybrid rice segment is well-known for its yield heterosis, P deficiency and bacterial leaf blight (BLB) diseases are the evident limitations. APMS6B, the female parent of DRRH-3 is susceptible to low P and bacterial blight disease. In the present study, the improvement of APMS6B to P starvation and resistance to bacterial leaf blight (BB) was carried out using marker-assisted backcross breeding (MABB) approach. Kasalath (+ *Pup1* QTL) was used as donor and a promising IL (ATR 594-1) at BC_1F_4 generation was identified with 81.15% RPGR. Concurrently, this IL was intercrossed with GU-2 (+ *Xa21* and *Xa38*). Hybridity of Intercross F_1s (ICF_1) was confirmed through foreground selection having maximum RPGR (88.29%) and were selfed to produce ICF_2. The resultant progenies were phenotyped for BB using *Xoo* inoculum (IX-020), simultaneously genotyped with gene specific functional SSR markers for *Xa21* and *Xa38*. The identified BB resistant plants were subjected to foreground selection for *Pup1*. Four promising ICF_3 plants (BP-10-1, BP-10-3, BP-10-5 and BP-10-15 with *Xa21, Xa38 and Pup1*) along with parents and checks were screened both in low P plot (<2 kg P_2O_5 ha^{-1}) as well as in normal plot (>25 kg P_2O_5 ha^{-1}) during *dry* and *wet* seasons 2018. Based on the field evaluation, four promising intercrossed lines were identified with better root architecture in terms of root length and root volume. In addition, less % reduction in grain yield (39.10%) under P starvation and less susceptibility indices values (<1) for BB were observed. These lines may be utilized in the CMS conversion programme and development of climate resilient, biotic and abiotic tolerant rice hybrids.

Key words: Rice, Low phosphorus tolerance, Bacterial Blight, maintainer line, APMS6B, MABB, hybrids.

INTRODUCTION

Rice is a major staple food crop for more than half of the world population and sole livelihood in Asian and African countries. Over the last few years, the yield potential of popular high yielding semi-dwarf rice varieties has encountered yield stagnation and further constrained by various biotic and abiotic stresses. Phosphorus deficiency is one of the least addressed and major yield limiting macro-nutrients in rice that steer to greater yield loss (Fageria and Baligar 1997; Rose et al. 2013). Globally, majority of soils (>50%) are P deficit and similarly Indian soils are either P deficient (49.3%) or medium level insufficiency (48.8%) or else possesses high P-fixing capacity and only 1.9% of soils are high in available P (Hasan 1996). Increased fertilizer costs and gradual depletion of rock phosphate, forced the country as the largest importer (90%) of P fertilizer (WEBECK et al. 2014). With increase in human population, reduction in arable lands and limited availability of fertilizer resources, sustainable rice production for diverse rice ecologies are major issues need to be addressed. There is an immense need to develop rice varieties and hybrids with enhanced P uptake efficiency to address P starvation tolerance (Heuer et al. 2017).

To overcome the P crisis, various agronomic and soil amelioration strategies are in force, however, sustainable, ecofriendly and long term approaches of breeding genotypes for low P is need of the hour. Genetic studies and screening of rice germplasm for P use efficiency and tolerance to low soil P is well documented (Fageria et al. 1988a; Fageria et al. 1988b; Wissuwa et al. 1998; Chin et al. 2011). A major QTL, *Pup1* associated with tolerance to low available soil P has been identified on chromosome 12 in an indica *aus* type landrace, Kasalath and was very well characterized (Wissuwa et al. 1998; Wissuwa et al. 2002; Heuer et al. 2009). The QTL *Pup1* has been fine-mapped and closely linked markers have been developed (Chin et al. 2009). A protein kinase gene *OsPSTOL1* was identified within *Pup1* QTL region, which promotes extensive root growth, enabling enhanced uptake of P from the soil. Many of the modern rice cultivars lack *Pup1* loci and thereby are susceptible to P starvation. Successful attempts were made to introgress *OsPSTOL1* through marker-assisted breeding into the elite rice varieties like IR-64, ASD 16, ADT 43, BPT 5204, Improved Samba Mahsuri (Swamy et al. 2020; Anila et al. 2018; Chin et al. 2011; Chithrameenal et al. 2018). Phenotypic evaluation of the introgression lines infers that *Pup1* is effective in different genetic backgrounds and has the potential to significantly enhance grain yield under irrigated and rainfed ecosystems of rice cultivation having low soil phosphorus (Chin et al. 2011).

Among the biotic stresses, bacterial blight (BB) is a serious disease of rice at maximum tillering stage and consequent yield loss of up to 80% (Mew 1987; Mew 1993). Chemical control being ineffective and environmentally hazardous, the development and introduction of resistant cultivars is the most economical, effective and eco-friendly method of controlling bacterial blight disease (Khush GS, Mackill DJ 1989; Ogawa 1993). Presently 46 bacterial blight resistance genes have been identified (Zhou et al. 2011; Kim et al. 2015) and available for genetic improvement of rice cultivar for bacterial blight disease. Pyramiding two or more BB resistant genes into a single rice variety helps to develop durable BB resistance rice varieties (Khush GS, Mackill DJ 1989).

Hybrid rice is a promising technology with exploitation of standard heterosis for yield and responds well to high inputs like chemical fertilizers. Three-line hybrid rice breeding programme in India have been successful by utilizing an elite wild-abortive (WA) cytoplasm (Senguttuvel et al. 2019) and till-date, 127 rice hybrids are released in India for commercial cultivation with a cultivable area of 3.5Mha. DRRH-3 is the first public bred medium

slender rice hybrid released for commercial cultivation in 2008 by ICAR-Indian Institute of Rice Research (ICAR-IIRR) (https://www.icar-iirr.org/). However, DRRH-3 encounters both abiotic and biotic stresses; especially bacterial blight and P deficiency which are of prime importance in India. Neither the CMS line (APMS6B) and the restorer line (RPHR 1005) nor does the derived hybrid (DRRH-3) acquire tolerance to low phosphorus and resistance to BB. In previous study, the improved maintainer line APMS6B of DRRH-3 known as GU-2, was developed by incorporating two bacterial leaf blight (BB) resistance genes (*Xa21* and *Xa38*), through marker assisted backcross breeding (MABB) approach (Yugander et al. 2018). The scope of marker-assisted breeding for targeted introgression of BB resistance genes (Yugander et al. 2018; Sundaram et al. 2008; Amante-Bordeos et al. 1992; Hittalmani et al. 2000) and *Pup1* (Swamy et al. 2020; Anila et al. 2018; Chin et al. 2011; Chithrameenal et al. 2018) has been successfully demonstrated. In all these studies, either BB resistance or low P tolerant genes (any two traits) were introgressed separately into commercial varieties. In the present study, we have attempted to stack *Pup*-1 from Kasalath and BB resistant genes from improved APMS6B line GU-2 using marker-assisted backcross breeding strategy coupled with phenotype-based selection. To the best of our knowledge, this is the first report on improvement of low P tolerance combined with bacterial leaf blight in hybrid rice maintainer line.

Materials and methods

Plant materials: Recurrent Parent - APMS6B

APMS6B, an elite, sturdy with medium slender (MS) grain maintainer line of DRRH-3 hybrid, was used as recurrent parent. The maintainer line has good combining ability and dwarf plant stature but is highly susceptible to BB and intolerant to low phosphorus soil.

Donor for *Pup1*: Kasalath

Kasalath, an Indian *aus* type land race adapted to poor soils contains beneficial allele for drought tolerance, phosphate-deficiency tolerance and early maturity (Gamuyao et al. 2012) was used as *Pup1* donor.

Donor for Bacterial Blight

GU-2 is a Near Isogenic Line of APMS6B, developed through marker assisted backcross breeding of two bacterial blight resistance genes *viz*., *Xa21* and *Xa38* to confer broad spectrum resistance to bacterial blight (Yugander et al. 2018) and has medium slender grain and excellent cooking quality which was used as donor parent for bacterial leaf blight disease. The parental lines *viz.,* APMS6B, GU-2, RPHR 1005 (male parent of DRRH-3) and hybrid DRRH-3 were intolerant to low soil P.

DNA markers for *Pup1* and BB and genotyping

DNA isolation from the parents, F_1s, and intercross-derived plants was carried out using a Miniprep protocol (Zheng KL. 1995). PCR protocol described by Chin et al. (2011) was adopted for the markers associated with *Pup*-1 (K46-1, 46-2) and BB gene *Xa21* specific marker, pTA248 and *Xa38* specific marker, Os04g5350-1 was followed as suggested by Ronald et al. (1992) and (Bhasin et al. 2012) respectively (Table-1). While for the amplification of rice SSR markers, the protocol described in Sundaram et al. (2008) was followed. The amplified products of K46-1, K46-2, pTA248 (*Xa21*) and Os04g5350-1 (*Xa38*) were electrophoretically resolved in 2% and 1.2% Seakem LE agarose gel (Lonza, USA), while the amplified fragments from SSR markers were resolved on 3.5% agarose gels. Recombinant selection was carried out with two parental polymorphic SSR markers, ESSR12-14.7 and ESSR12-

17.4, which are located at a physical distance of ~ 1.5–2.0 Mb from *Pup1* locus. Parental polymorphic SSR markers (n = 111) which were distributed evenly covering all the 12 chromosomes were used for background selection and to identify those positive plants, which have maximum recovery of the recurrent parent genome as described in Sundaram et al. (2008).

Breeding strategy for Marker-assisted transfer of *Pup1* and *Xa21* & *Xa38*

The detailed scheme of hybridization programme is shown in Figure-1. The maintainer line APMS6B was crossed with Kasalath. The true F_1s were confirmed with K46-1 marker and backcrossed with APMS6B. The resultant BC_1F_1 was further confirmed for hybridity with K46-1. Based on background selection, one line with maximum recovery of parental genome was selfed to produce BC_1F_2. These lines were screened under low P soil in field condition. Based on superior performance for agro morphological traits for low P, the line ATR 594-1, an introgression line of APMS6B and Kasalath was identified as improved donor for *Pup1*. Further, GU-2 (a donor for *Xa21* and *Xa38*) was crossed with ATR-594-1. At the time of initiation of the present work, GU-2 lines were not stabilized to use directly as donor with Kasalath. The true inter cross F_1s were identified with the help of the functional dominant markers, K46-1 and K46-2 (Chin et al. 2011). True intercross F_1 with maximum recovery of recurrent parent genome was selfed to get intercross F_2. ICF_2 plants were screened for bacterial blight resistance with inoculum of IXI020 available at ICAR-IIRR Hyderabad (Laha et al. 2009). Plants showing phenotypic resistance to BB and agronomic desirable plant type were screened for foreground selection for *Pup-1* and *Xa21, Xa38*. Plants showing positive for *Pup1*, *Xa21* and *Xa38* were selfed to get intercross F_3.

Phenotypic screening of intercross-derived lines for low P tolerance

Parental lines (APMS6B, GU-2, RPHR 1005 and DRRH3) and four ICF_3 selected lines were sown in normal soil. Thirty days old seedlings were then transplanted in low soil P (low soil phosphorus< 2 kg P_2O_5 ha^{-1}) plot of ICAR-IIRR, Hyderabad, Telangana state, India (Latitude 17.3201° N, Longitude 78.3939° E and 542m above MSL), at a spacing of 15 × 20 cm (in three rows, 10 hills per row) along with the donor and recurrent parents and grown until maturity. The plants were also grown in graded plots with normal soil P (> 25 kg P_2O_5 ha^{-1}) in three rows with 20 hills per row for comparison of performance. Standard agronomic practices were followed to raise a healthy crop in both normal and low P plots. Agro-morphological characters like days to 50% flowering, plant height, panicle length, number of tillers per plant, number of productive tillers, Spikelet fertility (%), 1000 seed weight, yield per plant, root length and root volume were recorded by adopting modified IRRI Standard Evaluation Scale (SES, 2002).

Root phenotyping of plants were carried out in low P and normal P plot of BC_1F_2 and ICF_2 generation. The plants were uprooted from field without damaging roots and washed with running water to remove soil for measuring root related parameters like root length and root volume. The average root length was measured from crown of the root to tip of the root, while the root volume was measured by using water displacement method, wherein the cleaned roots were placed in a measuring cylinder containing water and the rise in the level of water in the measuring cylinder was calculated by subtracting the level of water after placing the root in the measuring cylinder from the level of water before placing the root in the measuring cylinder which represented the root volume in the unit of milliliters (Swamy et al. 2019; Anila et al. 2018).

Phenotypic screening of intercrossed lines for Bacterial blight resistance

The parents and derived introgressed lines (ILs) were screened using bacterial culture of a virulent local isolate of the bacterial blight pathogen *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*), IX-020 (Hyderabad, Telangana state, India). *Xoo* strain was cultured and stored as described by Laha et al. (2009). The bacterial culture inoculated at maximum tillering stage by following leaf clip inoculation method described by Kauffman et al. (1973). Inoculum of bacterial suspension containing 10^{8-9} cfu/ml was maintained. The lesion length on leaves was measured at 15 days after inoculation. Scoring was done based on percent diseased leaf area by adopting modified IRRI Standard Evaluation Scale, 2002. A total of 46 BC_1F_3 and 380 ICF_2 plants along with the parents were screened for bacterial leaf blight disease.

Statistical analysis

The agro-morphological and phenotypic data pertaining to ILs and parents evaluated under graded P plot and BB incidence were analyzed by calculating mean, analysis of variance (ANOVA) and least significance difference (5% CD) by standard procedures (Gomez KA 1984) using *IndoStat version 9.2* software and excel software. The analysis of background genome recovery percentage was assessed using GGT-2.0 software.

Results

Introgression of *Pup1* into APMS6B

The low P sensitive female parent APMS6B was crossed with *Pup* 1 donor Kasalath and true F_1s were confirmed with *Pup1* specific PCR based dominant marker, K46-1. True positive nine F_1s were backcrossed with APMS6B to produce 23 BC_1F_1 plants. Genotyping of these 23 BC_1F_1 plants with *Pup1* specific marker K46-1 identified 12 positive plants. Co-dominant marker K20-1-1 (physical distance of 106 kb from the candidate gene for *Pup1*) further used for genotyping of the positive plants which revealed that all 12 plants were in heterozygous for *Pup1*. The 12 positive plants were then screened with parental polymorphic SSR markers (n=65) for identification of maximum recovery of the recurrent parent genome. Subsequently one plant with maximum recurrent parent genome (81.15%) and positive for *Pup1* was identified and was selfed to produce 183 BC_1F_2 plants.

The BC_1F_2 were phenotyped for yield traits under low P (<2 Kg P_2O_5 ha^{-1}) plot at Research farm, ICAR-IIRR, Hyderabad and also genotyped with five *Pup1* specific markers (K46-1, K46-2, K48, K52 and K59). Advancement of selected BC_1F_2 plants was carried out based on phenotypic similarity with APMS6B possessing *Pup1* loci. The 46 positive *Pup1* plants were advanced to BC_1F_3 and then to BC_1F_4. Eleven plants were positive for all five dominant markers, 17 plants positive for four dominant markers, 10 plants were positive for three dominant markers and eight plants were positive for two dominant markers. Along with the foreground markers for *Pup1*, the derived lines were also screened for negative selection for fertility restoration (*Rf*) using candidate gene specific markers DRRM-RF3-10 for *Rf3* and RMS-PPR-9-1 for *Rf4* (Figure 2). Eleven positive plants showed complete maintenance ability for fertility markers and showed a distinctive tolerance under low P in comparison with recurrent parent were finally selected, however all the 11 derived lines were susceptible to bacterial blight disease.

Introgression of BB genes into *Pup1* positive plant

Of the eleven *Pup1* positive BC_1F_4 individuals, the line ATR-594-1 is phenotypically superior among the introgressed lines, parents and checks under low soil P condition, and morphological similar to APMS6B and was

used as male parent for intercrossing with GU-2, a Near Isogenic Line (NIL) of APMS6B possessing *Xa21* and *Xa38* to obtain ICF_1 plants. A total of 25 ICF_1 plants were genotyped with dominant functional markers K46-1, K46-2 and nine of them were identified positive for the presence of *Pup1* locus (Figure 4). They were later subjected to background selection using 65 parental polymorphic SSR markers and the plant BP-10 showed highest recurrent parent genome recovery percent of 88.29% (Figure 3) was identified and selfed to produce ICF_2s.

Screening of intercrossed plants for bacterial blight resistance and marker assisted selection for *Pup1* and BB

A total of 380 ICF_2 plants along with the parents (APMS6B, GU-2 & Kasalath) were screened using virulent local strain of the bacterial blight pathogen, IX-020 (Hyderabad, Telangana state, India) of which 152 plants were found to be resistant with a lesion length of 1-4 cm and a score of 1-3 range. Susceptible checks, APMS6B and Kasalath showed susceptibility to the disease with a lesion length of >8 cm and disease score of 9, while resistant check APMS6B NIL (GU-2) was highly resistant to the disease with a lesion length of <1cm with a disease score of 1.

Phenotypically BB resistant 152 plants were genotyped for *Pup-1* markers, where 36 plants showed positive variation for dominant markers, however only nine plants were positive for all the three *Pup-1* specific dominant markers K46-1, K46-2 and K-52 (Figure 4) and also phenotypically similar to the recurrent parent, APMS6B. Later these nine plants were subjected to recombinant selection with ESSR12-14.7 and ESSR12-17.4 which flank *Pup-1* QTL on 12^{th} chromosome (Figure 5). Plants genotypically positive for *Pup1* were further subjected to genotyping with *Xa21* specific marker, pTA248 and *Xa38* specific marker, Os04g5350-1 (Figure 6). Four positive plants (*viz*., BP-10-1, BP-10-3, BP-10-5 and BP-10-15) possessing *Pup-1*, *Xa21* and *Xa38* gene combinations were forwarded to ICF_3.

Evaluation of parents and intercrossed lines for low phosphorus tolerance

Parental lines (APMS6B, GU-2 and Kasalath), checks for susceptibility (IR 64) and tolerance (Swarna) and four ICF_3 progeny (positive for three genes *Xa21*, *Xa38* and *Pup1*) lines were screened under two P levels *viz*., <2 kg P_2O_5 ha^{-1}(low P) and >25 kg P_2O_5 ha^{-1} (Normal condition) and the performance of them is presented in supplementary Figure 7.

Characterization of *Pup1* and BB positive introgression lines for yield and morphological traits

The agronomic performance of the intercross-derived lines in ICF_3 generation were on par with the recurrent parent for most of the traits examined (Table 2 and 3). The recipient parent, APMS6B recorded mean grain yield of 4.90 g/plant (low P) and 17.48 g/plant (normal), while the donor parent (*Kasalath)* recorded 8.07 g/plant (low P) and 15.29 g/plant (normal). The intercross derived lines *viz*., BP-10-1, BP-10-3, BP-10-5 and BP-10-15 had higher grain yields (8.61-11.90 g/plant in low P and 19.65-21.24 g/plant in normal) than the recurrent parent. Root length and root volume of improved lines showed higher values (15.75-24.50 cm and 15.00-32.50 ml) than recurrent parent APMS6B (7.67 cm and 8.33 ml). All the four selected lines showed phenotypic similarity with the recipient parent (APMS6B) (Figure 7).

Under low P condition, the improved lines with *Pup1* and *Xa21 & Xa38* recorded significant yield advantage over the recurrent parent APMS6B. These derived lines had exhibited a yield reduction by 43.99% to 56.65% comparatively lesser to APMS6B (71.97%). Under low P condition, the plant height of derived lines had an upper hand over the recurrent parent ranged between 64-68.25cm and increase in productive tillers from 6 to 11.5; panicle

length increased from 17.5 to 22.0 cm, spikelet fertility from 63.38 to 75.33%. There has been an increase in 1000 seed weight from 14.45 to 16.03g. The yield stability and stress susceptibility indices were higher in contrast to APMS6B *i.e.,* between 0.56- 0.43 and 0.76 -0.98 respectively (Table 4). Based on the agronomic performances of the introgressed lines it can be understood that under low P condition these lines had been effectively tolerant to low P levels and had been better in all yield traits compared to APMS6B.

Under normal soil P plot, there was non-significant difference found in *Pup1* introgressed lines in comparison with APMS6B for days to 50% flowering (84.33-94.67 days) and plant height (67.67- 74 cm). But we have an observed a remarkable difference in panicle length (20.83-21.67cm), an increase in the number of total tillers and productive tillers of 12.33-15.67 and 12.02-14.67 respectively. The spikelet fertility has gone up to 77.30 to 83.34% and an increase from 19 g to 19.62 g over 1000 seed weight. The major significant characters observed were the improvement in root length (16.50-22.67cm) and root volume (20.33-29.33ml) and yield per plant up to 19.65-21.24g. The distinctness, uniformity and stability (DUS) characters of introgressed lines were similar to recurrent parent.

Discussion

The outreach of hybrid rice technology in India and all over the rice growing states of Asia had tasted success in terms of heterotic yield when compared to inbred varieties. However, most of the commercial rice hybrids grown in India and elsewhere are highly vulnerable to many biotic and abiotic stresses; one among them is low soil P stress, where 49 % of Indian soils are deficit and 90% dependency in importing P based fertilizers (Hasan 1996; Swamy et al. 2019). Therefore, our attempt was to improve APMS6B, the maintainer line for its tolerance to low soil P by targeted introgression of *Pup1*, a major QTL from Kasalath and introgression of *Xa21* and *Xa38*, a major bacterial blight resistance gene, with a great inception for improvement of maintainer line among many of biotic and abiotic stresses through MABB.

Identification of Pup1 introgression lines in the background of APMS6B

The donor, Kasalath was initially identified in a screening of 30 diverse rice genotypes in a P-deficient soil in Japan under rain-fed conditions. The gene *OsPSTOL1* is known to enhance P uptake in rice (Wissuwa and Ae 2001; Chin et al. 2009). The phenotypic data derived from Nipponbare contrasting near isogenic lines (NILs) with and without the QTL showed that *Pup1* increases P uptake (Wissuwa et al. 2002; Wissuwa and Ae 2001) and confers a significant yield advantage (2 to 4fold) in pot experiments under different P-deficient soil types and environments (Chin et al. 2009). The *Pup1* derived donor ATR 594-1 is derivative of BC_1F_3 line of limited backcrossing of APMS6B with Kasalath (a perfect maintainer) facilitates adaptive introgression (Rieseberg LH. 1993) of more functional alleles of Kasalath and in turn enhances genetic diversity. The GU-2, (an improved version of APMS6B possessing *Xa21* & *Xa38*) was intercrossed with ATR 594-1. The approach of MABB involving foreground, recombinant and background selection was adopted in the study for quick transfer of *Pup1, Xa21* and *Xa38* into APMS6B, without any significant changes in the background genome and the number of backcross and intercross was limited to just two using MABB coupled with stringent phenotyping selection procedures. At each intercross

selfing generation, the dominant functional markers, K46-1 and K46-2 were used to monitor the segregation of *Pup1*. Two SSR markers, *viz.,* ESSR12-14.7 and ESSR12-17.4 were employed for recombinant selection.

Four promising ICF_3 lines with maximum of APMS6B genome to an extent of 87–89%, indicates the efficacy of stringent marker assisted backcross breeding and also the fact that limited backcrossing was a great success in the present study. In addition to deploying dominant, functional markers, i.e. K46-1and K46-2, to reconfirm the derived ICF_2 lines, we also employed a recombinant selection between the flanking marker and *Pup1*. ICF_2 plants showing phenotypically resistant to BB and *Pup*-1 positive were genotyped for *Xa21* specific marker, pTA248 and *Xa38* specific marker, Os04g5350-1. Four plants positive for all three loci i.e. *Pup-1* QTL and *Xa21* and *Xa38* genes were selected and selfed for generation advancement, these findings are in confirmation with (Chithrameenal et al. 2018; Swamy et al. 2020).

Identification of low P tolerant BB resistant lines

Bacterial blight is an important disease affecting rice cultivation in major rice-growing areas of Asia (Mew 1993) *Xa21* is a broad-spectrum resistance gene derived from *O. longistaminata* widely used in gene pyramiding programme (Gopalakrishnan et al. 2008; Sundaram et al. 2008; Basavaraj et al. 2010; Hari et al. 2011) The *Xa21* gene was introduced to cultivar PR106 in India because it showed resistance to 17 *Xoo* strains in Punjab, India (Singh et al. 2001). *Xa21* is considered as the most effective gene that show effective resistance to 88% of the *Xoo* strains in India (Mishra et al. 2013). However, evidence of virulent strains for *Xa21* for BB in India was also reported (Yugander et al. 2017; Mishra et al. 2013). Another BB gene *Xa38* provides a high level of resistance to most of the prevailing BB races of *Xoo* from Punjab state of India (Cheema et al. 2008). The combination of *Xa21* and *Xa38* in the selected advanced back cross derived lines (BDLs) exhibited broad-spectrum BB resistance when compared to the parental lines containing only *Xa21* developed in many earlier studies (Chen *et al.,* 2000; Hari *et al.,* 2011). Yugander et al. (2018) reported improved versions of the stable maintainer line, APMS6B, which possess a very high level of BB resistance (conferred by two major, dominant BB resistance genes *Xa21* and *Xa38*) and marginally higher yields as compared to the recurrent parent. BB resistance genes *Xa21* and *Xa13* along blast resistant genes *Pi2* and *Pi54* were also successfully introgressed in Pusa Basmati 1509 (Sagar et al. 2020). To develop durable BB resistance in one of the Indian elite rice variety Samba Mahsuri (Sundaram et al. 2008) introgressed *Xa 21, xa13 and xa 5.* The resultant introgressed variety know as Improved Samba Mahsuri became very much popular among rice farmers and now grown in an area of 2.5 Lakh hectare. All these studies stage the importance for development of disease resistance rice varieties in the crux. Several BB resistant genes have been mapped and a combination of two genes (*Xa21* and *Xa38*) is reported to be effective against bacterial blight isolates in major rice growing regions of the world. Incorporation of *Xa21* and *Xa38* genes together will pave resistance in a wider context in majority of prevailing races (Yugander et al. 2018). Our study demonstrated the efficiency of marker-assisted backcross breeding combined with phenotyping in developing rice genotypes with improved disease resistance and enhanced level of tolerance to P starvation.

Four breeding lines of improved APMS6B, possessing *Pup1 and Xa21 & Xa38* (*viz*., BP-10-1, BP-10-3, BP-10-5 and BP-10-15), which were very much similar to APMS6B in terms of agro-morphological traits, grain quality and possessing recurrent parent genome recovery ranging from 87 to 89%, were evaluated in a plot having optimum soil

P level and also in the low soil P plot, during *Kharif* 2018, which have most of the traits similar to or better than the original recurrent parent. Their advanced intercross–derived lines were not analyzed for the other genes/QTLs (such as drought tolerance), which might have contributed for significant improvement in few of the traits other than low soil P tolerance.

Use of molecular markers spread across the rice genome helped us in recovering desirable attributes like early flowering, medium slender grain type and high yield traits of APMS6B (with ~ 89% recurrent parent genome recovery) within one backcross and intercross thus saving time and resources in the present study. Similar investigations were published for marker-assisted gene pyramiding in different rice varieties for various stress conditions (Chithrameenal et al. 2018; Swamy et al. 2020).

The introgressed lines had a delayed flowering under low P compared to normal soil conditions, however earlier than original APMS6B parent. This could be the adaptive mechanism of plants for low P to provide maximum yield and biomass (Swamy et al. 2020) (Nord & Lynch. 2008). Generally, plants respond to nutrient stress by shortening its life cycle, delay flowering to attain threshold nutrient uptake for flowering and less biomass and few number seeds. Due to increased uptake of P and better utilization by *Pup1* introgressed lines makes to recover phenological instability noticed in low P condition (Wissuwa et al. 2002; Chin et al. 2011; Gamuyao et al. 2012). Under the low soil P condition, significant improvement in plant height, panicle length, total and productive tillers, spikelet fertility, root length, root volume and grain yield *per se* among the *Pup1* introgressed lines relative to the recipient parent APMS6B (Swamy et al. 2020; Anila et al. 2018; Magalhaes et al. 2017; Chithrameenal et al. 2018). However, no significant difference for yield and associated traits noticed in introgressed lines and perform on par with recurrent parent APMS6B under normal soil phosphorus condition. The presence of phosphorus responsive gene *OsPSTOL1 (Pup1)* in introgressed lines facilitates the effectual root system like root length and root volume which in turn helps in improvement of grain yield under P deficit soil as reported by Gamuyao et al. (2012) and Yugandhar et al. (2017). Positive and significant association of root length and volume in derived lines noticed in terms of tolerance to low P and grain yield and similar observations were reported by earlier workers (Kale et al. 2021). The *Pup1* possessed lines with modified root architecture and morphology facilitate exploration and adaptive mechanism to access P beyond the rhizosphere when bioavailable P is limited under low P plot.

Selection of introgressed lines based on percent yield reduction and stress susceptibility were lesser than APMS6B but yield stability and stress tolerance index calculated was higher than APMS6B (Yugander et al. 2018). A single line BP10-15 performed better with respect to number of productive tillers per plant, panicle length, spikelet fertility, 1000 seed weight and grain yield per plant in both normal and low soil P plots.

Improving parental lines for phosphorous tolerance and high P usage efficiency can be a breakthrough for reduced usage of phosphate fertilizers. Such development not only reduces the cost of cultivation but also the development of hybrids that yields more under acidic, alkaline soils where low P conditions are frequently noticed. The improved maintainers for *Pup1* tolerance and BB resistance can be used as donors for future breeding programme. Agricultural sustenance in rice production with heterotic yield can be achieved by MABB with the improved hybrid DRRH-3. Improvement of parental lines in rice for biotic and abiotic stress tolerance will be an additional uplift for hybrid rice cultivation in stress prone unfavorable environments.

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Author Contribution Statement

MN and SP conceived, planned and designed study. MN, Be.P, VJ, NP and MY conducted experiments. MN and SP analyzed the data and wrote the manuscript. AMS, KKB, KMB, LGS, GC, GR, ASH, Br.P, TMD and MKR critically edited the manuscript. SRM helped in coordination of the study and edited final draft of the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Legends of Tables and Figures

Table 1. Details on markers used in foreground selection for identification of phosphorus starvation tolerance and bacterial blight resistance.

Table 2: Evaluation of ICF_3 lines (positive for *Pup*-1, *Xa*-21 and *Xa*-38 genes) in Normal soil P plot at ICAR-IIRR, during, *Kharif* -2018.

Table 3. Evaluation of ICF_3 lines (positive for ***Pup*-1, *Xa*-21 and *Xa*-38 genes**) in low soil P plot at ICAR-IIRR during Kharif-2018

Table 4: Stress indices calculated for *Pup*-1 introgressed APMS-6B ICF_3 lines based on per plant yield under normal and Low soil P conditions.

Figure 1: Crossing plan followed for the introgression of Pup-1 and Xa21+ Xa38 into APMS6B.

Figure 2: Genotyping of BC1F3 derived lines from cross APMS6B and Kasalath for Rf4 and Rf3 gene specific markers.

Figure 3: Graphical representation of Background selection on ICF2

Figure 4: Foreground selection for Pup1 among ICF2 plants using Pup1 specific markers K46-1, K46-2 and K-52.

Figure 5: Recombinant selection of Pup1 positive ICF2 plants with the flanking marker ESSR12-14.7 and ESSR-12-17.4

Figure 6: Genotypic selection for Bacterial blight disease among ICF2 plants using Xa21 and Xa38 gene specific markers such as pTA-248 and Oso4g53050-1, respectively.

Figure 7: A. Performance of Pup1 introgressed lines under low P (<2 kg P2O5 ha-1) and **B.** Normal conditions (>25 kg P2O5 ha-1); **C.** Root length of parents and ILs under low P condition; **D.** Panicles of parents and Pup1 introgressed lines; E. Scoring of BB inoculated leaf.

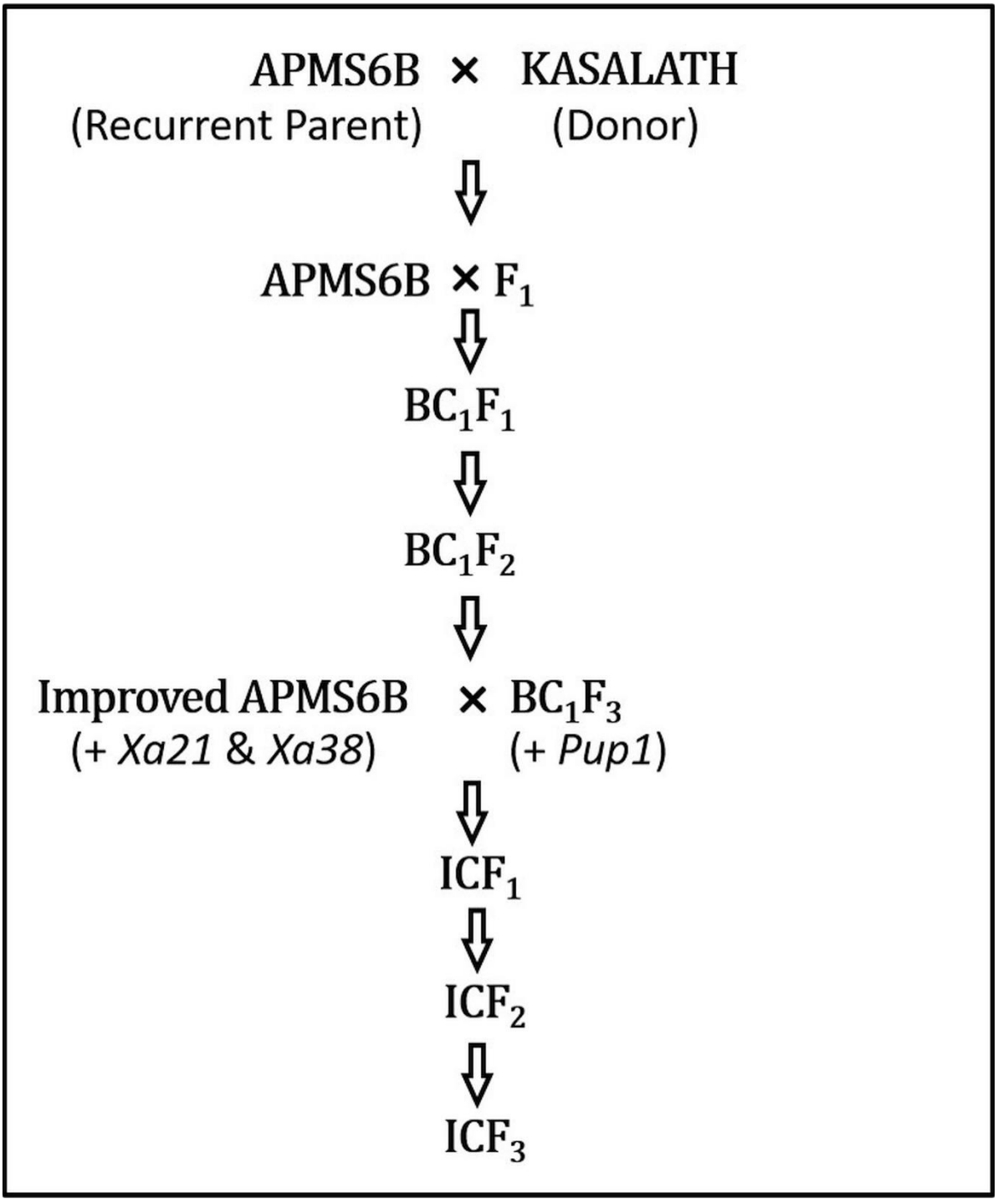
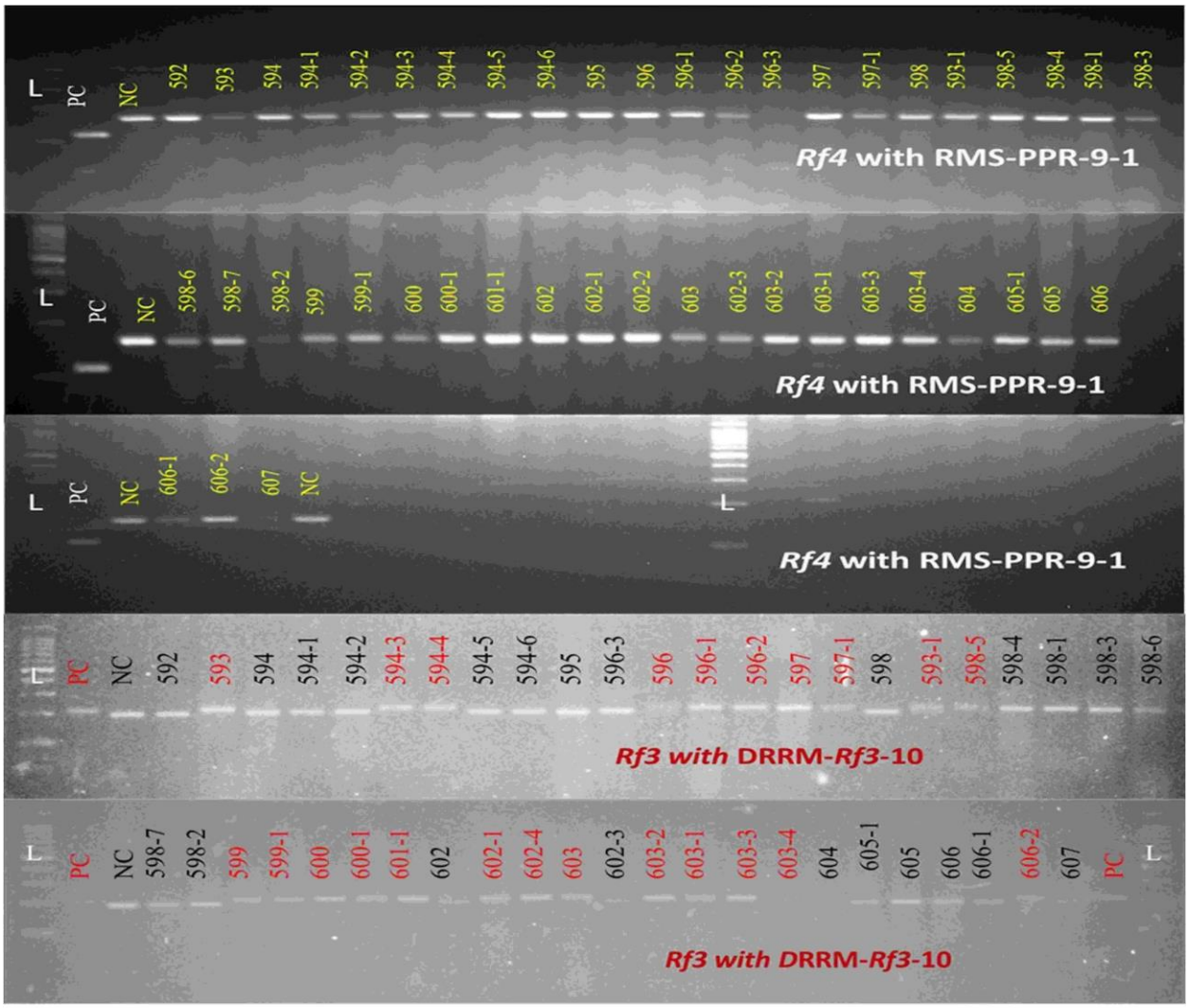
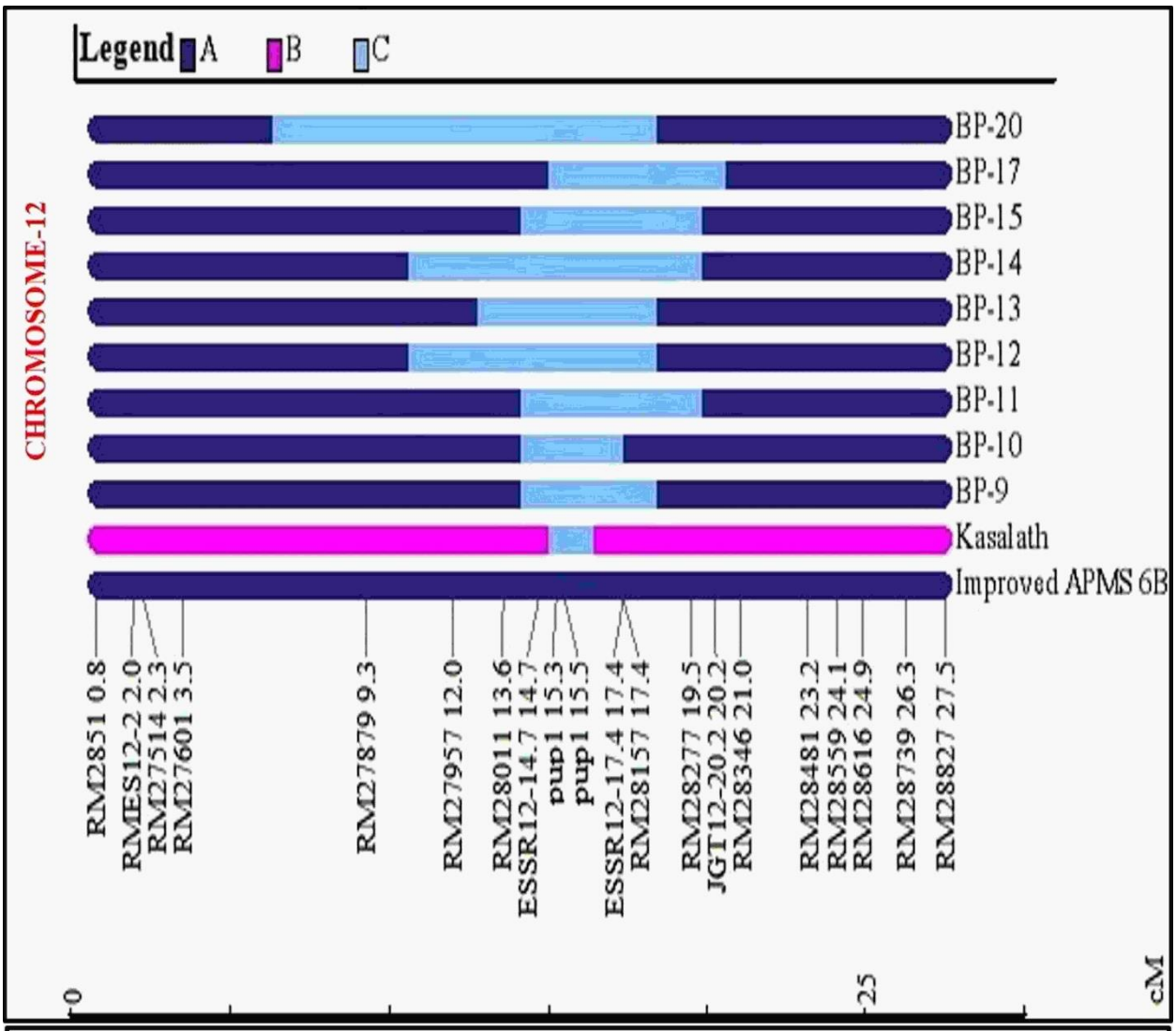


Figure 1: Crossing plan followed for the introgression of Pup-1 and Xa21+ Xa38 into APMS6B.



L– 100 bp ladder, **PC**– positive check (KMR-3R) and **NC**- negative check (APMS-6B); Numbers– $BC_1F_{3\,S}$ derived lines from cross APMS-6B and Kasalath. Yellow and black colour font in entries indicates the absence of respective restoration of fertility (*viz.*, *Rf4* with RMS-PPR-9-1 and *Rf3* with DRRM-*Rf3*-10, respectively) and red colour entries indicate presence of DRRM-*Rf3*-10 in genotypes.

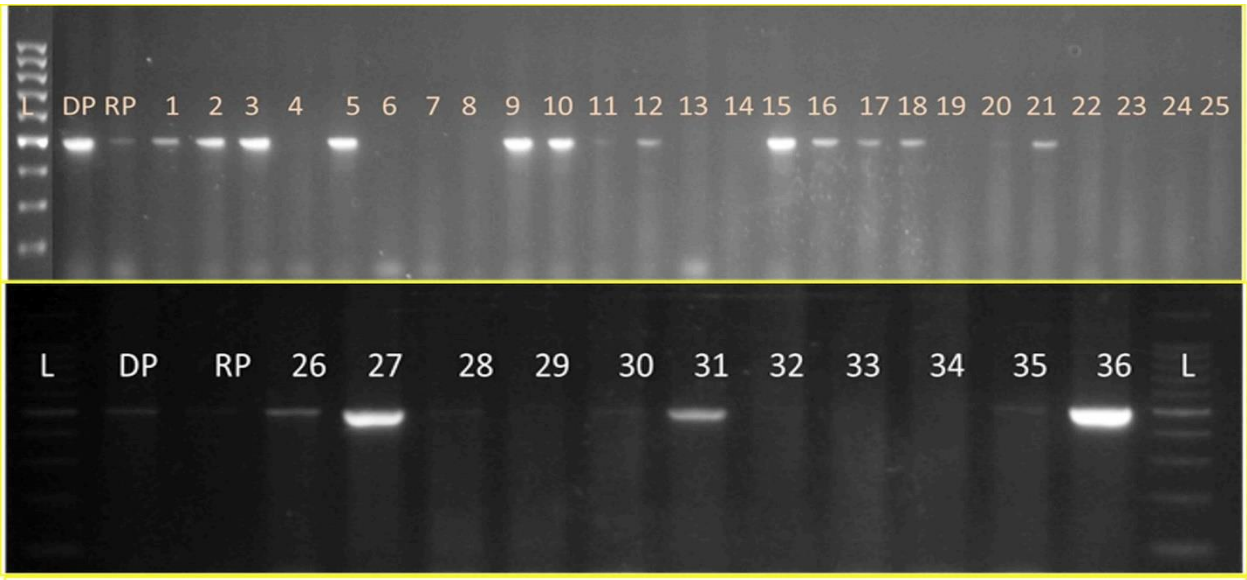
Figure 2: Genotyping of BC1F3 derived lines from cross APMS6B and Kasalath for Rf4 and Rf3 gene specific markers.



A)- Improved APMS6B; B)- Kasalath; C)- *Pup*-1 Locus

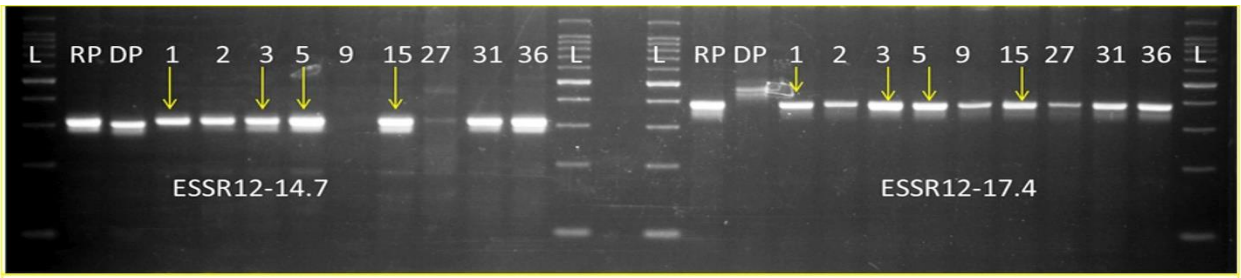
The ICF_1 plants BP-9, BP-10, BP-11, BP-12, BP-13, BP-14 BP-15, BP-17 and BP-20 had a varying per cent of genome recovery. The plant BP-10 which had maximum genome recovery (88.29%) of recurrent parent Improved APMS6B genome was selected and selfed to produce ICF_2.

Figure 3: Graphical representation of Background selection on ICF2



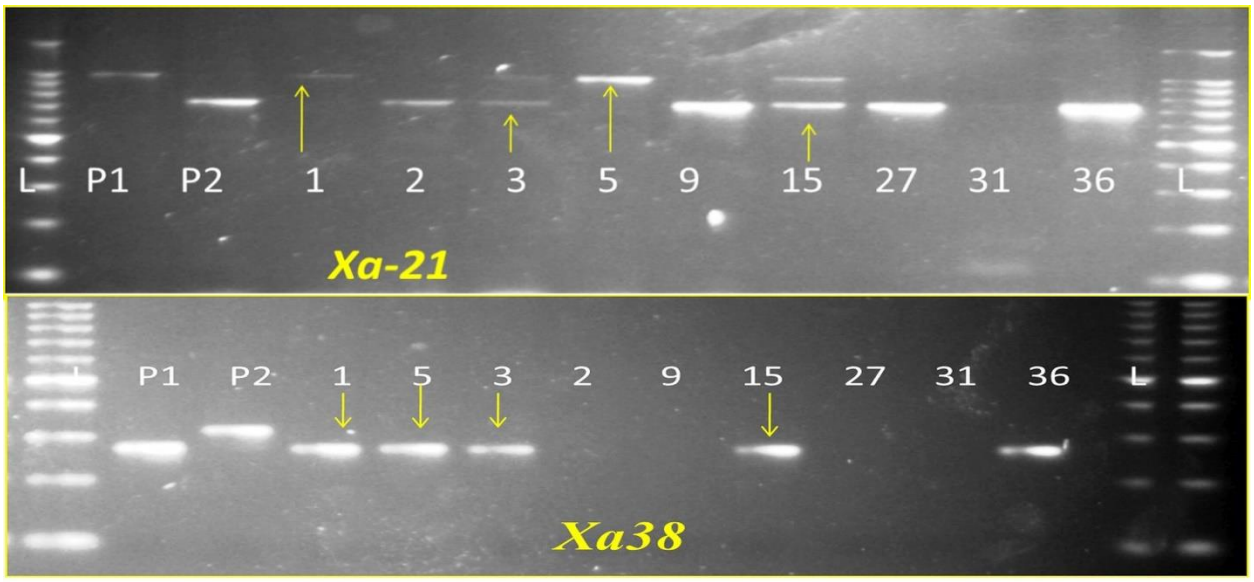
L– 100 bp ladder, DP– Kasalath (Donor), RP– GU-2 (APMS6B NILs) (Recipient), Presence of bands indicates ICF_2 possessing *Pup-1*

Figure 4: Foreground selection for Pup1 among ICF2 plants using Pup1 specific markers K46-1, K46-2 and K-52.



L– 100 bp ladder, RP– GU-2 (APMS-6B NILs), DP– Kasalath, 1 to 36 – ICF_1 plants and Arrows indicate homozygous for APMS-6B locus flanking the QTL nearby

Figure 5: Recombinant selection of Pup1 positive ICF2 plants with the flanking marker ESSR12-14.7 and ESSR-12-17.4



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511 **Figure 6:** Genotypic selection for Bacterial blight disease among ICF2 plants using Xa21 and Xa38 gene specific
512 markers such as pTA-248 and Oso4g53050-1, respectively.

513

A. Performance of *Pup1* introgressed lines under low P (<2 kg P_2O_5 ha^{-1}) and B. Normal conditions (>25 kg P_2O_5 ha^{-1}); C. Root length of parents and ILs under low P condition; D. Panicles of parents and *Pup1* introgressed lines; E. Scoring of BB inoculated leaf

Figure 7: A. Performance of Pup1 introgressed lines under low P (<2 kg P2O5 ha-1) and **B.** Normal conditions (>25 kg P2O5 ha-1); **C.** Root length of parents and ILs under low P condition; **D.** Panicles of parents and Pup1 introgressed lines; E. Scoring of BB inoculated leaf.

520 **Table 1.** Details on markers used in foreground selection for identification of phosphorus starvation tolerance and bacterial blight resistance

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522

S.No.	Trait	Marker Name	Sequence	Chr. No	Marker position	Annealing temperature	Amplicon size	Reference
1	*Pup-1*	K46-1	F TGAGATAGCCGTCAAGATGCT R AAGGACCACCATTCCATAGC	12	275,7102 to 276,232	62	523	Chin et al., 2011
3	*Pup-1*	K46-2	F AGGAAGATGGTTGTCGTTGG R TTCACACCAAACAGTGTTGTC	12	276,371 to 276,597	62	227	
4	*Pup-1*	K-48	F CAGCATTCAGCAAGACAACAG R ATCCGTGTGGAGCAACTCATC	12	282,795 to 283,640	62	847	
5	*Pup-1*	K-52	F ACCGTTCCCAACAGATTCCAT R CCCGTAATAGCAACAACCCAA	12	300,870 to 301,374	62	505	
6	*Pup-1*	K-59	F GGACACGGATTCAAGGAGGA R TGCTTTCCATTTGCGGCTC	12	324,843 to 325,392	62	550	
7	*Xa21*	pTA 248	F AGACGCGGGAAGGGTGGTTCCCGGA R AGACGCGGGTAATCGAAAGATGAAA	11	1,758,862 to 1,759,369 (1.75Mb)	55	950/650	Ronald *et al*., 1992
8	*Xa38*	Oso4g53050-1	F TCTTCTATTGCTAACATTGGTG R TCGCATTCATTTTCAGAG R CTCCACCAGTGCAGGTTTT	4L	38.4-kb	56	269/317	Bhasin *et al.*, 2012

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525 **Table 2:** Evaluation of ICF_3 lines (positive for *Pup*-1, *Xa*-21 and *Xa*-38 genes) in Normal soil P plot at ICAR-IIRR, during, *Kharif* -2018.

526

Genotypes	Days to 50% Flowering	Plant Height (cm)	Panicle Length (cm)	Total tillers per plant	Productive tillers per plant	Spikelet Fertility (%)	1000 Seed Weight (g)	Yield per Plant (g)	Root length (cm)	Root volume (ml)
BP-10-1	84.33	73.67	21.67	12.67	12.17	80.34	19.11	20.75	16.50	26.00
BP-10-3	92.00	67.67	20.83	15.67	14.67	81.36	19.00	19.65	22.67	29.33
BP-10-5	94.67	74.00	21.50	12.67	12.17	77.30	19.22	20.77	19.00	20.33
BP-10-15	91.33	72.33	21.17	12.33	12.02	83.34	19.62	21.24	19.00	22.67
APMS6B	98.00	69.33	20.33	11.66	11.33	73.33	16.18	17.48	13.67	16.33
APMS6B NIL (GU2)	96.00	71.00	19.55	12.33	12.00	76.52	16.94	18.33	16.00	20.00
Kasalath	86.67	98.33	21.50	12.00	10.67	79.50	18.88	15.29	22.00	17.33
Swarna	111.00	80.93	21.00	11.00	9.33	85.04	21.85	18.74	14.00	18.00
IR-64	91.00	82.88	17.08	9.67	8.67	83.94	21.77	18.59	21.33	27.33
CV	2.06	2.00	5.78	7.22	7.27	4.49	6.47	10.25	10.63	10.79
CD @ 5%	3.24	2.57	1.99	1.48	1.4	6.04	2.08	3.26	3.26	3.97

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528

Table 3. Evaluation of ICF_3 lines (positive for ***Pup*-1, *Xa*-21 and *Xa*-38 genes**) in low soil P plot at ICAR-IIRR during Kharif-2018

Genotypes	Days to 50% Flowering	Plant Height (cm)	Panicle Length (cm)	Productive tillers per plant	Total tillers per plant	Spikelet Fertility (%)	1000 Seed Weight (g)	Yield per Plant (g)	Root length (cm)	Root volume (ml)
BP-10-1	96.33	64.00	17.50	9.00	9.33	75.33	15.22	10.32	24.50	32.50
BP-10-3	98.33	64.00	17.75	11.50	11.50	63.38	15.20	8.61	19.50	15.00
BP-10-5	98.00	65.50	22.00	6.50	7.00	69.16	16.03	9.00	15.75	19.50
BP-10-15	99.00	68.25	18.50	6.00	8.00	71.27	14.45	11.90	17.00	25.00
APMS6B	105.00	61.67	17.33	5.67	6.00	58.30	12.77	4.90	7.67	8.33
APMS6B NIL (GU2)	103.00	64.33	16.25	6.00	7.00	62.20	13.79	5.80	21.83	21.67
Kasalath	98.00	86.22	16.33	9.44	9.56	69.40	17.01	8.07	20.08	17.67
Swarna	117.33	64.67	17.17	6.33	8.67	77.04	17.53	8.05	11.67	16.33
IR-64	111.08	51.00	13.00	4.00	4.67	72.18	17.33	5.01	19.00	23.33
CV	2.76	3.74	6.23	14.19	15.21	6.67	9.18	10.63	7.01	24.16
CD @ 5%	4.77	4.11	1.81	1.71	2.03	7.69	2.38	1.42	2.05	8.08

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535 **Table 4:** Stress indices calculated for *Pup*-1 introgressed APMS-6B ICF_3 lines based on per plant yield under normal and Low soil P conditions.
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Genotype	NORMAL P	P STRESS/ LOW P	STI/ stress tolerance index	SSI/ stress susceptibility index	YSI/ yield stability index	% Yield reduction
BP-10-1	20.75	10.32	0.59	0.87	0.50	50.26
BP-10-3	19.65	8.61	0.47	0.97	0.44	56.17
BP-10-5	20.77	9.00	0.52	0.98	0.43	56.65
BP-10-15	21.24	11.90	0.70	0.76	0.56	43.99
APMS6B	17.48	4.90	0.24	1.24	0.28	71.97
GU-2	18.33	5.80	0.29	1.18	0.32	68.38
Kasalath	15.29	8.07	0.34	0.81	0.53	47.23
Swarna	18.74	8.05	0.42	0.98	0.43	57.05
IR-64	18.59	5.01	0.26	1.26	0.27	73.05

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