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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Abstract  Phosphorus (P) is one of the macronutrients essential for plant growth and development. Rice ($Oryza sativa$ L.) is sensitive to P starvation and its deficiency influences many key plant functions, resulting in crop yield penalties. Although the hybrid rice segment is well-known for its yield heterosis, P deficiency and bacterial leaf blight diseases are 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 the marker-assisted backcross breeding approach. Kasalath (+$Pup1$ QTL) was used as a donor, and a promising IL (ATR 594-1) at BC$_1$F$_4$ generation was identified with 81.15% of recurrent parent genome recovery. Concurrently, this IL was intercrossed with GU-2 (+$Xa21$ and $Xa38$). Intercross F$_1$ (ICF$_1$) hybridity was confirmed through foreground selection having maximum RPGR (88.29%) and was selfed to produce ICF$_2$. The resultant progenies were phenotyped for BB, 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$_3$ lines (BP-10-1, BP-10-3, BP-10-5, and BP-10-15) along with parents and checks were screened both in low P plot (<2 kg P$_2$O$_5$ ha$^{-1}$) as well as in normal plot (>25 kg P$_2$O$_5$ ha$^{-1}$) during dry and wet seasons 2018. The field evaluation identified four promising intercrossed lines with better root growth in the primary root length of extracted zone and root volume. In addition, fewer reductions in grain yield (39.10%) under P starvation and less susceptibility indices values (<1) for BB were observed. These lines may be exploited in the CMS conversion and development of climate-resilient, biotic and abiotic stress-tolerant rice hybrids.

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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 is 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, most soils (> 50%) are 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 the gradual depletion of rock phosphate forced the country to be the largest importer (90%) of P fertilizer (Webeck et al. 2014). With the increase in human population, reduction in arable lands, and limited availability of fertilizer resources, sustainable rice production for diverse rice ecologies are major issues that 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, eco-friendly, and long-term approaches of breeding genotypes for low P are the need of the hour. Genetic studies and screening of rice germplasm for P use efficiency and tolerance to low soil P are well documented (Fageria et al. 1988a, b; Wissuwa et al. 1998; Chin et al. 2011). A major QTL, Pup1 associated with tolerance to low available soil P, was identified on chromosome 12 in an indica aus type landrace, Kasalath, and was very well characterized (Wissuwa et al. 1998; 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 the Pup1 QTL region, promoting extensive root growth enabling enhanced P uptake from the soil. Many modern rice cultivars lack Pup1 loci and are susceptible to P starvation. Successful attempts were made to introgress OsPSTOL1 through marker-assisted breeding into the elite rice varieties like IR-64, ASD16, ADT 43, BPT 5204, Improved Samba Mahsuri (Chin et al. 2011; Anila et al. 2018; Chithra-meenal et al. 2018; Swamy et al. 2020). Phenotypic evaluation of the introgression lines infers that Pup1 is effective in different genetic backgrounds and can 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 the maximum tillering stage and consequent yield loss of up to 80% (Mew 1987; 1993). Chemical control being ineffective and environmentally hazardous, developing and introducing resistant cultivars is the most economical, effective, and eco-friendly method of controlling bacterial blight disease (Khush and Mackill 1989; Ogawa 1993). Presently 46 bacterial blight resistance genes have been identified (Zhou et al. 2011; Kim et al. 2015) and are available for genetic improvement of rice cultivar for bacterial blight disease. Pyramid-ing two or more BB-resistant genes into a single rice variety helps to develop durable BB resistance rice varieties (Khush and Mackill 1989).

Hybrid rice is a promising technology that exploits standard heterosis for yield and responds well to high inputs like chemical fertilizers. Three-line hybrid rice breeding in India has successfully utilized an elite wild-abortive (WA) cytoplasm (Senguttuvel et al. 2019). To date, 127 rice hybrids have been released in India for commercial cultivation with a cultiva-ble 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 important in India. Neither the CMS line (APMS6B) and the restorer line (RPHR-1005R) nor does the derived hybrid (DRRH-3) acquire tolerance to low phosphorus and resistance to BB. In a 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 (Amante-Bordeos et al. 1992; Hittalmani et al. 2000; Sundaram et al. 2008; Yugander et al. 2018) and \textit{Pup1} (Chin et al. 2011; Anila et al. 2018; Chithrameenal et al. 2018; Swamy et al. 2020) 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 \textit{Pup1}, \textit{Xa21}, and \textit{Xa38} genes in the background of APMS6B using a marker-assisted backcross breeding strategy coupled with phenotype-based selection.

**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 the 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 \textit{Pup1}:** Kasalath

Kasalath, an Indian \textit{aus} type landrace adapted to poor soils, contains beneficial alleles for drought tolerance, phosphate-deficiency tolerance, and early maturity (Gamuyao et al. 2012) was used as a \textit{Pup1} donor.

**Donor for bacterial blight**

GU-2 is a Near Isogenic Line (NIL) of APMS6B, developed through marker-assisted backcross breeding of two bacterial blight resistance genes viz., \textit{Xa21} and \textit{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-1005R (male parent of DRRH-3), and hybrid DRRH-3 were sensitive to low soil P.

**DNA markers for \textit{Pup1} and BB and genotyping**

DNA isolation from the parents, F1s, and intercross-derived plants were done using a Miniprep protocol (Zheng 1995). PCR was carried out for the \textit{Pup1} specific markers, K4-1 and K46-2 as per the conditions described in Chin et al. (2011). Concerning the markers specific for \textit{Xa21} (pTA248) and \textit{Xa38} (Os04g5350-1), PCR was done as per the procedure described in 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 (Lonz, USA), while the amplified fragments from SSR markers were resolved on 3.5% agarose gels. The recombinant selection was carried out with two parental polymorphic SSR markers, ESSR12-17.4 and ESSR12-17.4, located at a physical distance of ~1.5 to 2.0 Mb from the \textit{Pup1} locus. Parental polymorphic SSR markers (n=111), 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 the marker-assisted transfer of \textit{Pup1}, \textit{Xa21} and \textit{Xa38}**

The detailed scheme of hybridization is shown in Fig. 1. The maintainer line APMS6B was crossed with Kasalath. The true F1s were confirmed with the K46-1 marker and backcrossed with APMS6B. The resultant BC1F1 was further confirmed for hybridity with K46-1. Based on background selection, one line with maximum recovery of the parental genome was selfed to produce BC1F2. These lines were screened under low P soil in field conditions. Based on superior performance for agro-morphological traits for low P, ATR 594-1, an introgression line of APMS6B, and Kasalath were identified as the improved donor for \textit{Pup1}.

Further, GU-2 (a donor for \textit{Xa21} and \textit{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 a donor with Kasalath. The true intercross F1s were identified with the help of the dominant functional markers, K46-1 and K46-2 (Chin et al. 2011). True intercross F1 with maximum recovery of recurrent parent genome was selfed to get intercross F2. ICF2 plants
were screened for bacterial blight resistance with an 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 \( Pup1 \), \( Xa21 \) and \( Xa38 \). Plants showing the

| S. no. | Trait | Marker Name | Sequence | Chr. no. | Marker position | Annealing temperature | Amplicon size | References |
|--------|-------|-------------|----------|----------|-----------------|-----------------------|--------------|------------|
| 1      | Pup1  | K46-1       | F        | GAGATAGCC GTCAAGATG CT | 12 | 275,710–276,232 | 62 | 523 | Chin et al. (2011) |
|        |       |             | R        | AAGGACCAC CATCCCATG GC |                |                        |              |            |
| 3      | Pup1  | K46-2       | F        | AGGAAGATG GTTGTCGGT GG | 12 | 276,371–276,597 | 62 | 227 |            |
|        |       |             | R        | TTCACACCA AACAGTGTT GTC |                |                        |              |            |
| 4      | Pup1  | K-48        | F        | CAGCATTTCA GCAAGACAA CAG | 12 | 282,795–283,640 | 62 | 847 |            |
|        |       |             | R        | ATCCGCGGTG GAGCAAACCT ATC |                |                        |              |            |
| 5      | Pup1  | K-52        | F        | ACCGTTCCTAC AAGATTTCA CAT | 12 | 300,870–301,374 | 62 | 505 |            |
|        |       |             | R        | CCCGTAATA GCAACAACC CAA |                |                        |              |            |
| 6      | Pup1  | K-59        | F        | GGACACCGGA TTCAAGGAG GA | 12 | 324,843–325,392 | 62 | 550 |            |
|        |       |             | R        | TGCTTTCCA TTTCGGCCTC |                |                        |              |            |
| 7      | Xa21  | pTA 248     | F        | AGACGCAGAG AAGGGTGGTT CCCGGA | 11 | 1,758,862–1,759,369 (1.75 Mb) | 55 | 950/650 | Ronald et al. (1992) |
|        |       |             | R        | AGACGCAGGA TAAATCAGAA GATGAAA |                |                        |              |            |
| 8      | Xa38  | Oso4g53050-1 | F        | TCTTCTATT GCTAACATT GGTG | 4L | 38.4 kb | 56 | 269/317 | Bhasin et al. (2012) |
|        |       |             | R        | TGCATTCA TTTCAGAG |                |                        |              |            |
|        |       |             | R        | CTCCACCACT GCAAGG TTTT |                |                        |              |            |
presence of Pup1, Xa21, and Xa38 genes were selfed to get intercross F3.

Phenotypic screening of intercross-derived lines for low P tolerance

Parental lines (APMS6B, GU-2, RPHR-1005R, and DRRH3) and four ICF3 selected lines were sown in normal soil. Thirty days old seedlings were then transplanted in low soil P (low soil phosphorus < 2 kg P₂O₅ ha⁻¹) plot of ICAR-IIRR, Hyderabad, Telangana state, India (Latitude 17.3201° N, Longitude 78.3939° E and 542 m above MSL), at a spacing of 15 × 20 cm (in three rows, ten 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 P₂O₅ ha⁻¹) in three rows with 20 hills per row for performance comparison. To raise a healthy crop in both normal and low P plots, standard agronomic practices were followed. Agromorphological characters like days to 50% flowering, plant height, panicle length, number of tillers per plant, number of productive tillers, Spikelet fertility (%), 1000-grain weight, grain yield per plant, primary root length of extracted zone, and root volume were recorded by adopting modified IRRI Standard Evaluation Scale (SES 2002).

At the panicle initiation stage, root phenotyping of plants was carried out in low P and normal P plots of BC1F2 and ICF2 generation. Plants were first tagged with a label, and then from the crown of the plant, 20 cm diameter of a circle in soil was marked and with iron rod loosen soil up to 20 cm depth and scoop out rhizosphere. The plants were uprooted from the field without damaging the primary root and washed with running water to remove soil adhering to roots for measuring root-related parameters like primary root length and root volume. The average primary root length was measured from the crown of the root to the primary root of the extracted zone. Root volume was measured by the upper displacement of the water method. Here, the cleaned roots were placed in a measuring cylinder containing water. The rise in the level of water in the measuring cylinder was calculated, which is represented as root volume in the unit of milliliters (Anila et al. 2018; Swamy et al. 2019).

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 was inoculated at the maximum tillering stage following the leaf clip inoculation method described by Kauffman et al. (1973). Inoculum of bacterial suspension containing 10⁸–⁹ cfu/ml was maintained. The lesion length on leaves was measured at 15 days after inoculation. Scoring was done based on the percent diseased leaf area by adopting the modified IRRI Standard Evaluation Scale (2002). A total of 46 BC1F3 and 380 ICF2 plants along with the parents were screened for bacterial leaf blight disease.

Statistical analysis

The agro-morphological and phenotypic data of 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.
Results

Introgression of Pup1 into APMS6B

True positive nine F1s were confirmed with Pup1 specific PCR-based dominant marker, K46-1, and backcrossed with APMS6B to produce 23 BC1F1 plants. Genotyping these 23 BC1F1 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) was further used for genotyping the positive plants, revealing that all 12 plants were heterozygous for Pup1. The 12 positive plants were then screened with parental polymorphic SSR markers (n = 65) to identify 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 BC1F2 plants.

The BC1F2 were phenotyped for yield traits under low P (< 2 kg P2O5 ha−1) plot at ICAR-IIRR Research farm, Hyderabad, and also genotyped with five Pup1 specific markers (K46-1, K46-2, K48, K52, and K59). Advancement of selected BC1F2 plants was carried out based on phenotypic similarity with APMS6B possessing Pup1 loci. The 46 positive Pup1 plants were advanced to BC1F3 and then to BC1F4. Eleven plants were positive for all five dominant markers, 17 plants positive for four dominant markers, ten 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 (Fig. 2). Eleven positive plants showed complete maintenance ability for fertility restoration markers and showed a distinctive tolerance under low P compared with a recurrent parent were finally selected; however, all the 11 derived lines were susceptible to bacterial blight disease.

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

Of the eleven Pup1 positive BC1F4 individuals, the line ATR-594-1 is phenotypically superior among the introgressed lines, parents, and checks under low soil P condition and morphologically similar to APMS6B and was used as the male parent for intercrossing with GU-2, a NIL of APMS6B possessing Xa21 and Xa38 to obtain ICF1 plants. A total of 25 ICF1 plants were genotyped with dominant functional markers K46-1, K46-2 and nine of them were identified as positive for the presence of Pup1 locus. They were later subjected to background selection using 65 parental polymorphic SSR markers. The plant BP-10 showed the highest recurrent parent genome recovery percent of 88.29% (Fig. 3) was identified and selfed to produce ICF2s.

A total of 380 ICF2 plants along with the parents (APMS6B, GU-2 and Kasalath) were screened using a 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. In comparison, resistant check APMS6B NIL (GU-2) was highly resistant to the disease with a lesion length of < 1 cm 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 K52 (Fig. 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 the 12th chromosome (Fig. 5). Plants genotypically positive for Pup1 were further subjected to genotyping with Xa21 specific marker, pTA248, and Xa38 specific marker, Os04g5350-1 (Fig. 6). Four positive plants (viz., BP-10-1, BP-10-3, BP-10-5, and

Introgression of BB genes into Pup1 positive plant
BP-10-15) possessing *Pup1*, *Xa21*, and *Xa38* gene combinations were forwarded to ICF₃.

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 ICF₃ progeny (positive for three genes *Xa21*, *Xa38*, and *Pup1*) lines were screened under two P levels viz., < 2 kg P₂O₅ ha⁻¹ (low P) and > 25 kg P₂O₅ ha⁻¹ (normal) and their performance of them are presented in Fig. 7.

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

The agronomic performance of the intercross-derived lines in ICF₃ generation was on par with the recurrent parent for most of the traits examined (Table 2).

The recipient parent, APMS₆B recorded a 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. Primary root length of extracted zone 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 four selected lines showed phenotypic similarity with the recipient parent (APMS₆B) (Fig. 7).

Under low P conditions, the improved lines with *Pup1*, *Xa21* and *Xa38* recorded a significant yield advantage over the recurrent parent APMS₆B. These derived lines had exhibited a yield reduction by 43.99–56.65%, comparatively lesser to APMS₆B (71.97%). Under low P condition, the plant height
**Fig. 3** Graphical representation of Background selection on ICF$_2$

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$_2$.

**Fig. 4** Foreground selection for Pup1 among ICF$_2$ plants using Pup1 specific markers K46-1, K46-2, and K-52

L– 100 bp ladder; DP– Kasalath (Donor), RP– GU-2 (APMS6B NILs) (Recipient), Presence of bands indicates ICF$_2$ possessing Pup-1.
Fig. 5 Recombinant selection of *Pup1* positive ICF2 plants with the flanking marker ESSR12-14.7 and ESSR-12-17.4.

Fig. 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.

Fig. 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 Primary root length of the extracted zone of parents and ILs under low P condition; D Panicles of parents and *Pup1* introgressed lines; E Scoring of BB inoculated leaf.
of derived lines had the upper hand over the recurrent parent ranged between 64 and 68.25 cm and increased 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-grain weight from 14.45 to 16.03 g. The yield stability and stress susceptibility indices were higher in contrast to APMS6B i.e., between 0.56 and 0.43 and 0.76–0.98, respectively (Table 3). Based on the agronomic performances of the introgressed lines, it can be understood that under low P conditions, these lines had been effectively tolerant to low P levels and had been better in all yield traits than APMS6B.

Under normal soil P plot, there was a non-significant difference found in Pup1 introgressed lines compared to APMS6B for days to 50% flowering (84.33–94.67 days) and plant height (67.67–74 cm). But we have observed a remarkable difference in panicle length (20.83–21.67 cm), 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 77.30–83.34% and increased from 19 to 19.62 g over 1000-grain weight. The major significant characteristics observed were the improvement in primary root length of the extracted zone (16.50–22.67 cm) and root volume (20.33–29.33 ml), and grain yield per plant up to

| Genotypes            | Days to 50% flowering | Plant height (cm) | Panicle length (cm) | Productive tillers per plant | Total tillers per plant |
|----------------------|-----------------------|-------------------|---------------------|-------------------------------|-------------------------|
|                      | L P       | N P       | L P      | N P       | L P     | N P      | L P     | N P      | L P      | N P      |
| BP-10-1              | 96.33     | 84.33     | 64       | 73.67     | 17.5    | 21.67    | 9       | 12.67    | 9.33     | 12.17    |
| BP-10-3              | 98.33     | 92        | 64       | 67.67     | 17.75   | 20.83    | 11.5    | 15.67    | 11.5     | 14.67    |
| BP-10-5              | 98        | 94.67     | 65.5     | 74        | 22      | 21.5     | 6.5     | 12.67    | 7        | 12.17    |
| BP-10-15             | 99        | 91.33     | 68.25    | 72.33     | 18.5    | 21.17    | 6       | 12.33    | 8        | 12.02    |
| APMS6B               | 105       | 98        | 61.67    | 69.33     | 17.33   | 20.33    | 5.67    | 11.66    | 6        | 11.33    |
| APMS6B NIL (GU2)     | 103       | 96        | 64.33    | 71        | 16.25   | 19.55    | 6       | 12.33    | 7        | 12       |
| Kasalath             | 98        | 86.67     | 86.22    | 98.33     | 16.33   | 21.5     | 9.44    | 12       | 9.56     | 10.67    |
| Swarna               | 117.33    | 111       | 64.67    | 80.93     | 17.17   | 21       | 6.33    | 11       | 8.67     | 9.33     |
| IR-64                | 111.08    | 91        | 51       | 82.88     | 13      | 17.08    | 4       | 9.67     | 4.67     | 8.67     |
| CV                   | 2.76      | 2.06      | 3.74     | 2         | 6.23    | 5.78     | 14.19   | 7.22     | 15.21    | 7.27     |
| CD @ 5%              | 4.77      | 3.24      | 4.11     | 2.57      | 1.81    | 1.99     | 1.71    | 1.48     | 2.03     | 1.4      |
| Genotypes            | Spikelet fertility (%) | 1000-grain weight (g) | Grain yield per plant (g) | Primary root length of the extracted zone (cm) | Root volume |
|                      | L P       | N P       | L P      | N P       | L P     | N P      | L P     | N P      | L P | N P |
| BP-10-1              | 75.33     | 80.34     | 15.22    | 19.11     | 10.32   | 20.75    | 24.5    | 16.5     | 32.5 | 26   |
| BP-10-3              | 63.38     | 81.36     | 15.2     | 19        | 8.61    | 19.65    | 19.5    | 22.67    | 15   | 29.33 |
| BP-10-5              | 69.16     | 77.3      | 16.03    | 19.22     | 9       | 20.77    | 15.75   | 19       | 19.5 | 20.33 |
| BP-10-15             | 71.27     | 83.34     | 14.45    | 19.62     | 11.9    | 21.24    | 17      | 19       | 25   | 22.67 |
| APMS6B               | 58.3      | 73.33     | 12.77    | 16.18     | 4.9     | 17.48    | 7.67    | 13.67    | 8.33  | 16.33 |
| APMS6B NIL (GU2)     | 62.2      | 76.52     | 13.79    | 16.94     | 5.8     | 18.33    | 21.83   | 16       | 21.67 | 20    |
| Kasalath             | 69.4      | 79.5      | 17.01    | 18.88     | 8.07    | 15.29    | 20.08   | 22       | 17.67 | 17.33 |
| Swarna               | 77.04     | 85.04     | 17.53    | 21.85     | 8.05    | 18.74    | 11.67   | 14       | 16.33 | 18    |
| IR-64                | 72.18     | 83.94     | 17.33    | 21.77     | 5.01    | 18.59    | 19      | 21.33    | 23.33 | 27.33 |
| CV                   | 6.67      | 4.49      | 9.18     | 6.47      | 10.63   | 10.25    | 7.01    | 10.63    | 24.16 | 10.79 |
| CD @ 5%              | 7.69      | 6.04      | 2.38     | 2.08      | 1.42    | 3.26     | 2.05    | 3.26     | 8.08  | 3.97  |

L P Low P, N P Normal P
19.65–21.24 g. The distinctness, uniformity, and stability (DUS) character of introgressed lines were similar to the 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% dependent on importing P based fertilizers (Hasan 1996; Swamy et al. 2019). Therefore, we attempted 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 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 screening 30 diverse rice genotypes in P-deficient soil in Japan under rain-fed conditions. The gene \( OsPSTOL1 \) enhances P uptake in rice (Wissuwa and Ae 2001; Wissuwa et al. 2002) and confers a significant yield advantage (2–4-fold) 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\(_1\)F\(_3\) line of limited backcrossing of APMS6B with Kasalath (a perfect maintainer) facilitates adaptive introgression (Rieseberg 1993) of more functional alleles of Kasalath and, in turn, enhances genetic diversity. The GU-2 (an improved version of APMS6B possessing \( Xa21 \) and \( Xa38 \)) 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 reconfirm the derived ICF\(_2\) lines, we also employed a recombinant selection between the flanking marker and \( Pup1 \). ICF\(_2\) plants showing resistance reaction to IX020 and \( Pup1 \)-positive phenotypically were genotyped for \( Xa21 \) specific marker, pTA248.

**Table 3** Stress indices calculated for \( Pup-1 \) introgressed APMS-6B ICF\(_3\) lines based on grain yield per plant (g) under normal and Low P conditions

| Genotype   | Grain yield per plant (g) | Stress tolerance index (STI) | Stress susceptibility index (SSI) | Yield stability index (YSI) | Yield reduction (%) |
|------------|--------------------------|------------------------------|---------------------------------|-----------------------------|---------------------|
|            | Normal P | Low P |                          |                             |                       |                     |
| 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               |
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 (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 and Africa (Mew 1993) Xa21 is a broad-spectrum resistance gene derived from O. longistaminata widely used in gene pyramiding (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 the most effective gene that shows 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 (Mishra et al. 2013; Yugander et al. 2017). Another BB gene Xa38 provides a high level of resistance to most of the prevailing BB races of Xoo from the 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 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 (conferring 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 and 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 with Xa21, xa13 and xa 5 and the resultant introgressed variety is known as Improved Samba Mahsuri became popular among rice farmers and is now grown in an area of 0.25 million hectares. All these studies stage the importance of developing disease resistance rice varieties in the crux. Several BB resistant genes have been mapped, and a combination of two genes (Xa21 and Xa38) is effective against bacterial blight isolates in major rice-growing regions of the world. Incorporating Xa21 and Xa38 genes together will pave resistance in a wider context in a 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, Xa21, and 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 to significant improvement in a few of the traits other than low soil P tolerance.

The 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).

However, the introgressed lines had delayed flowering under low P than normal soil conditions and earlier than the original APMS6B parent. This could be the adaptive mechanism of plants for low P to provide maximum yield and biomass (Swamy et al. 2020). Generally, plants respond to nutrient stress by shortening their life cycle, delaying flowering to attain threshold nutrient uptake for flowering, and less biomass and few seeds. Due to increased P uptake and better utilization by Pup1 introgressed lines makes to recover phenological instability noticed in low P conditions (Wissuwa et al. 2002; Chin et al. 2011; Gamuyao et al. 2012). Under the low soil P condition, significant improvement was noticed in plant height, panicle length, total and productive tillers, spikelet fertility, primary root length of extracted zone, root volume, and grain yield perse among the
Pup1 introgressed lines relative to the recipient parent APMS6B (Magalhaes et al. 2017; Anila et al. 2018; Chithrameenal et al. 2018; Swamy et al. 2020). However, there is no significant difference in yield, and associated traits noticed in introgressed lines and performed on par with recurrent parent APMS6B under normal soil phosphorus conditions. The presence of phosphorus responsive gene OsPSTOL1 (Pup1) in introgressed lines facilitates the effectual root system like primary root length of extracted zone 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). A positive and significant association of the primary root length of extracted zone and volume in derived lines was noticed for tolerance to low P and grain yield. Earlier workers reported similar observations (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 for the number of productive tillers per plant, panicle length, spikelet fertility, 1000-grain weight, and grain yield per plant in 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 reduces the cost of cultivation and the development of hybrids that yield more under acidic, alkaline soils where low P conditions are frequently noticed. The improved maintainers with Pup1 tolerance and BB resistance can be used as donors for future breeding. MABB can achieve agricultural sustenance in rice production with heterotic yield and improved 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 MN and SP Conceived, planned and designed the study. MN, BeP, VJ, NP and MY Conducted the experiments. MN and SP Analyzed the data and wrote the manuscript. AMS, KKB, KMB, LGS, GC, GR, ASH, BrP, TMD and MKR Critically edited the manuscript. SP and SRM Helped in the coordination of the study and edited the final draft of the manuscript.

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