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RESEARCH

Marker-Assisted Introgression of *qSPP2.2* Loci from *Oryza longistaminata* Improves Spikelet Number in Basmati Rice

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Abstract

Basmati rice is the premium, export-quality, long, slender grains with pleasant aroma. Punjab Basmati 3 (PB3), a unique aromatic rice, is sensitive to photoperiod, semi-dwarf, tolerant to lodging, and resistant to bacterial blight (BB). However, it yields less than *Indica* varieties, mostly due to lower number of grains per panicle (GPP), which varies between 60 and 80. To increase the GPP in PB3, we transferred the grain number QTL *qSPP2.2* from *O. longistaminata* (A. Chev. et Roehr.) derived introgression line, RIL127 into PB3 using marker-assisted selection (MAS). Marker- assisted foreground selection using polymorphic markers linked to the *qSPP2.2* loci in plants from backcross progenies and their advanced self-progenies, coupled with their background analysis for BB resistance (*Xa21*, *xa13)*, Basmati aroma (*Badh2*) and intermediate amylose content (*wx* locus) and analysis of their grain quality characteristics ensured an efficient and successful introgression of the QTL *qSPP2.2* into PB3. The phenotypic expression of *qSPP2.2* in the Basmati background was studied in BC_1F_2, BC_2F_3 and their advanced selfed progenies, and it shows a 35–40% increase in the grain number of PB3, demonstrating a significant improvement of grain yield of the introgressed lines. Together, a large number of backcross progenies and their advanced self-progenies, coupled with MAS for desirable alleles, and extensive phenotyping have resulted in an improved version of PB3 with higher spikelet per panicle (SPP). Correlation analysis using 384 replicated F_2 plants revealed no significant correlation between SPP and grain length, however, a significant weak negative correlation (-0.150*) was observed between SPP and grain breadth, suggesting the possibility of raising the GPP and the total output of Basmati rice having exceptional quality.

Keywords Marker-assisted backcrossing · Markers-assisted selection · *Oryza longistaminata* · Punjab Basmati 3 · *qSPP2.2* · Spikelet per panicle

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Introduction

Rice (*Oryza sativa* L.) is the most important staple food crops and provides > 21% of the daily intake of calories to one third of the global population (Wang and Li 2008; Fitzgerald et al. 2009; Muthayya et al. 2014; Ito 2019). Globally, India is the second largest producer and consumer of rice after China. These two countries together account for > 50% of the global production and consumption of rice (Maraseni et al. 2018; www.icra.in2024). But there has been a deficit in the global rice production since the market year 2021-22 and India's share in global rice trade declined to around 34%, which is estimated to decline further, causing the global rice prices to remain high (www.icra.in2024). Basmati rice grown in the North-western part of Indo-Gangetic plains of the Indian subcontinent is highly valuable

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in the global market mainly because of its exceptional fragrance and distinct flavor, extra-long slender grains which elongate at least twice upon cooking and excellent eating quality characteristics (Singh et al. 1988; Singh 2000; Jhang et al. 2006).

The traditional Basmati cultivars are tall, highly photoperiod sensitive, nutrient unresponsive, and susceptible to various biotic stresses, resulting in low grain yield (Bhatia et al. 2011; Singh et al. 2023). Since the fiscal year 2019, the export volume of Basmati rice from India has steadily decreased (www.statista.com 2024). Improving the grain yield of the traditional Basmati varieties is challenging through conventional breeding due to the high degree of divergence between Basmati and *Indica* rice varieties, polygenic control of aroma and cooking quality traits, and loss of quality characteristics upon sexual hybridization (Singh et al. 2000, 2023; Mahajan et al. 2018). Therefore, marker-assisted selection (MAS) has become valuable tool for monitoring introgression into the genetic background of elite Basmati varieties (Jhang et al. 2006; Sagar et al. 2020; Singh et al. 2023).

Punjab Basmati 3 (PB3) is an improved version of Basmati 386 developed through MAS (Bhatia et al. 2011; Singh et al. 2014). It is a photoperiod-sensitive, semi-dwarf, and lodging-tolerant variety that grows a height of 105–110 cm and matures in about 139 days after sowing (DAS). Notably, it is also the first Basmati variety to exhibit resistance to BB (Bhatia et al. 2011; Singh et al. 2014). PB3 has long, slender, and translucent grains with strong aroma and appealing taste. These attributes contribute to its good cooking quality and palatability. Grains remain non-sticky and soft upon cooking (Singh et al. 2014). PB3 produces lower yield of 4.08 tons/hectare (16 quintals per acre) compared to *Indica* rice, primarily due to fewer grains per panicle (grains/panicle). However, PB3 yields nearly 30.88% higher than its one of the parents, Basmati 386 (3.11 tons/hectare). PB3 bears an average 335 panicles/square meter and 67 spikelets/panicle with a fertility rate of 87.8%. Additionally, the 1000 grain weight of PB3 is approximately 28.0 g, which is higher than that of *indica* varieties (Bhatia et al. 2011; Singh et al. 2014).

The rice grain yield/plant is a complex quantitative trait determined by four major parameters, namely panicles/plant, spikelet per panicle (SPP), grain weight and spikelet fertility. Among these, SPP is a highly variable component and shows the largest range of variation across rice accessions, making it a key trait for yield enhancement in the rice breeding programs (Li et al. 1997; Xing and Zhang 2010; Zuo and Li 2014; Kaur et al. 2018). SPP is a crucial component for increasing grain yield and consists of the number of spikelets with primary and secondary panicle branches (Xing and Zhang 2010). The ideal plant architecture model suggests that the future rice breeding should focus on developing larger panicles with 200–250 grains per panicle and reducing tillering (Khush 1995, 2005; Lu et al. 2022). Enhancing the yield of Basmati rice has been challenging, primarily due to the complex nature of its grain quality traits and limited compatibility with other rice genotypes (Khush and Dela Cruz 1998). It is also not clear whether the low grain number is negatively correlated with grain length or if these traits are independent. Thus, unravelling the genetic basis of SPP is essential for breeding high-yielding Basmati varieties.

The primary determinants of SPP in rice are the panicle architecture and branch differentiation, which are strongly linked to the phytohormone pathways and vascular development (Duan et al. 2019; Deveshwar et al. 2020). The first grain number-associated gene identified in rice was *Grain number 1a* (*Gn1a*)/CKX2 (Cytokinin oxidase 2), which was isolated through map-based cloning (Ashikari et al. 2005). Thereafter, many genes/QTLs for grain number/grain yield in rice have been mapped and characterized. For example, grain number QTLs, *qgpa7* (Tian et al. 2006), *qSPP1*, *qSPP2*, *qSPP3 and qSPP7* (Zhang et al. 2009), *qGN4-1*(Deshmukh et al. 2010), *qSPP6* (Kim et al. 2012), *FRIZZLE PANICLE* (*FZP*), *OsEPFL6*, *OsEPFL7*, *OsEPFL8*, and *OsEPFL9* negatively regulate SPP (Wang et al. 2023), whereas, *OsEPFL8* also controls rice spikelet fertility (Guo et al. 2023); grain size and weight QTLs, *qsmg11* (Fang et al. 2016), *qGL3.1* (Qi et al. 2012), *qGL7* (Bai et al. 2010), *GS2* (Zhang et al. 2013), *GS3* (Fan et al. 2006; Takano-Kai et al. 2009), *GW5* (Wan et al. 2008; Weng et al. 2008), *GW9* (Wen et al. 2024), *qSW5* (Shomura et al. 2008), *qGW2* (Song et al. 2007), *qTGW3.2* (Shao-qing et al. 2013), *qgw8.1* (Xie et al. 2006); panicle number QTLs, *qpnn1* (Obara et al. 2004), *qMOC1*(Li et al. 2004). Some of these QTLs/genes have been pyramided into elite varieties to enhance rice grain yield.

At Punjab Agricultural University (PAU), Ludhiana, India, an introgression line, IL1792, developed from the cross *O. sativa* cv.PR114/*O.longistaminata* IRGC104301B//3*PR114 was used for mapping the spikelet per panicle QTL, *qSPP2.2* (Kaur et al. 2018). The QTL, *qSPP2.2*, explaining about 30% of the phenotypic variability for SPP was mapped on chromosome 2 in a 167.1 kb region flanked by two SSR markers, RM13742 and RM13750 (Kaur et al. 2018). Gene annotation study revealed that three genes viz., *LOC_Os02g45010*, *LOC_Os02g44860 and LOC_Os02g44990* were the putative candidates controlling SPP (Kaur et al. 2018). The introgression line, IL1792 was again crossed to PR114 and a RIL population was generated. The introgression of *qSPP2.2* into BC_2F_6 and its subsequent backcross and advanced progenies showed a significantly higher SPP (mean SPP was >183) with a moderate tiller

number per plant (7–8 tillers/plant) than the recurrent parent PR114 (mean SPP > 143) and most of the Indian traditional and elite Basmati varieties (SPP varies between 60 and 80) (Bhatia et al. 2011; Singh et al. 2014; Kaur et al. 2018). One of the RILs, designated as RIL127 had the QTL allele from *O. longistaminata* and also exhibited higher SPP, hence selected for crossing with PB3 to produce F_1 progeny.

Wild species and landraces of rice are valuable sources for many agronomically important QTLs/genes, which could be harnessed to enhance rice productivity (Khush et al. 1977; Dalmacio et al. 1995; Brar and Singh 2011; Shakiba and Eizenga 2014; Bhatia et al. 2017). However, transferring a QTL from wild rice into a cultivated elite Basmati rice for improving agronomic traits like SPP is quite innovative, but challenging, mainly because of the complex nature of Basmati grain quality traits and limited compatibility with wild species. Therefore, a stable introgression line was initially developed using a standard non-basmati variety (PR114) and evaluated across multiple generations (Kaur et al. 2018). Finally, here we report a successful introgression of the *qSPP2.2* loci from the *O. longistaminata*-derived introgression line into the semi-dwarf and BB resistant Basmati variety PB3 through MAS. This study demonstrates that the QTL *qSPP2.2* could increase SPP when transferred to the genetic background of PB3 and also explores the inheritance and correlation of grain size and grain number in Basmati rice.

Materials and Methods

Plant Materials and Crossing Programs

Punjab Basmati (PB3) is an improved version of Basmati 386 and possesses long, slender grains, strong aroma, semi-dwarf stature, and resistance to bacterial leaf blight (pedigree: Basmati 386/IET 17948//Basmati 386*2). It was crossed with *O. longistaminata* (2n = 24, AA, Acc. IRGC 104301B) derived introgression line, RIL127 (derived from the cross *O. sativa* cv.PR114 /*O. longistaminata* IRGC104301B //3*PR114) carrying the *qSPP2.2* allele from *O. longistaminata* to produce F_1. The RIL127 exhibits mean SPP of 198.8. The PB3 was used as a female parent while RIL127 was used as male parent in this study. As many as 10 selected true F_1 plants (based on the selected polymorphic marker for the target QTL) were backcrossed to PB3 to produce BC_1F_1 seeds and self-fertilization was allowed to generate F_2 seeds during the year 2014. The marker-assisted foreground selection in backcross progenies and their advanced self-progenies, background analysis for important Basmati and grain quality characteristics, including BB resistance and phenotypic expression study of *qSPP2.2* in the background of Basmati rice, were done during the cropping seasons of the years 2015–2019. The overall breeding strategy followed in the marker-assisted introgression of the QTL *qSPP2.2* is presented in Fig. 1. The field experiments were conducted in the experimental area of School of Agricultural Biotechnology, Punjab Agricultural University (PAU), Ludhiana, India and off-season experimental area at National Rice Research Institute (NRRI), Cuttack, Odisha, India.

Studying Correlation Between the Number of Grains Per Panicle (GPP) and Grain Size in Basmati rice

Correlation analysis was performed to study if any negative correlation exists between grain size and grain number in Basmati rice. An F_2 population comprising 384 individuals from the cross PB3 x RIL127 was planted in a randomized block design (RBD) in the year 2015 at PAU, Ludhiana, India. After 20 days of transplanting, the tillers of each F_2 plant were detached into two and planted as replications. The identity of each F_2 plant was maintained strictly. The parents PB3 and RIL127 were also replicated in the same manner. The crop was raised following the recommended practices for Basmati rice. Data on the agronomic and yield component traits viz., days to 50% flowering (DF), plant height (PH), number of tillers per plant (TPP), number of panicles per plant (PPP), spikelet per panicle (SPP), sterile grains per panicle (SGP), fertile grains per panicle (FGP), grain length and breadth (GL & GB), L/B ratio and grain weight per plant (GWP) were recorded as per Standard Evaluation System for rice as described by International Rice Research Institute (SES 2013) on each of the replicated F_2 plant including both the parental lines. Data of SPP were recorded on three panicles in each plant, whereas GL and GB were recorded on 10 grains in each plant. The PH data was recorded when plants were at complete maturity. The GL and GB (mm) and their L/B ratio were recorded using a CanoScan grain scanner. Two-tailed Student's t-test and analysis of variance (ANOVA) were carried out using GraphPad Prism (v8.0.1). Correlation analysis was performed using the "metan" package in R (R Core Team 2018).

Marker-Assisted Introgression of *qSPP2.2* Loci into Punjab Basmati 3 (PB3)

Isolation of Genomic DNA and PCR Amplification Profile

The genomic DNA of the recipient/recurrent parent (PB3), donor parent (RIL127), F_1s and their backcrossed progenies were isolated by following standard CTAB method as described by Saghai-Maroof et al. (1984) with some minor modifications. The quality and integrity of DNA was assessed using 0.8% agarose gel. The samples were also quantified using NANODROP 8000 (Thermo Scientific, USA) spectrophotometer. The DNA of each sample was normalized to

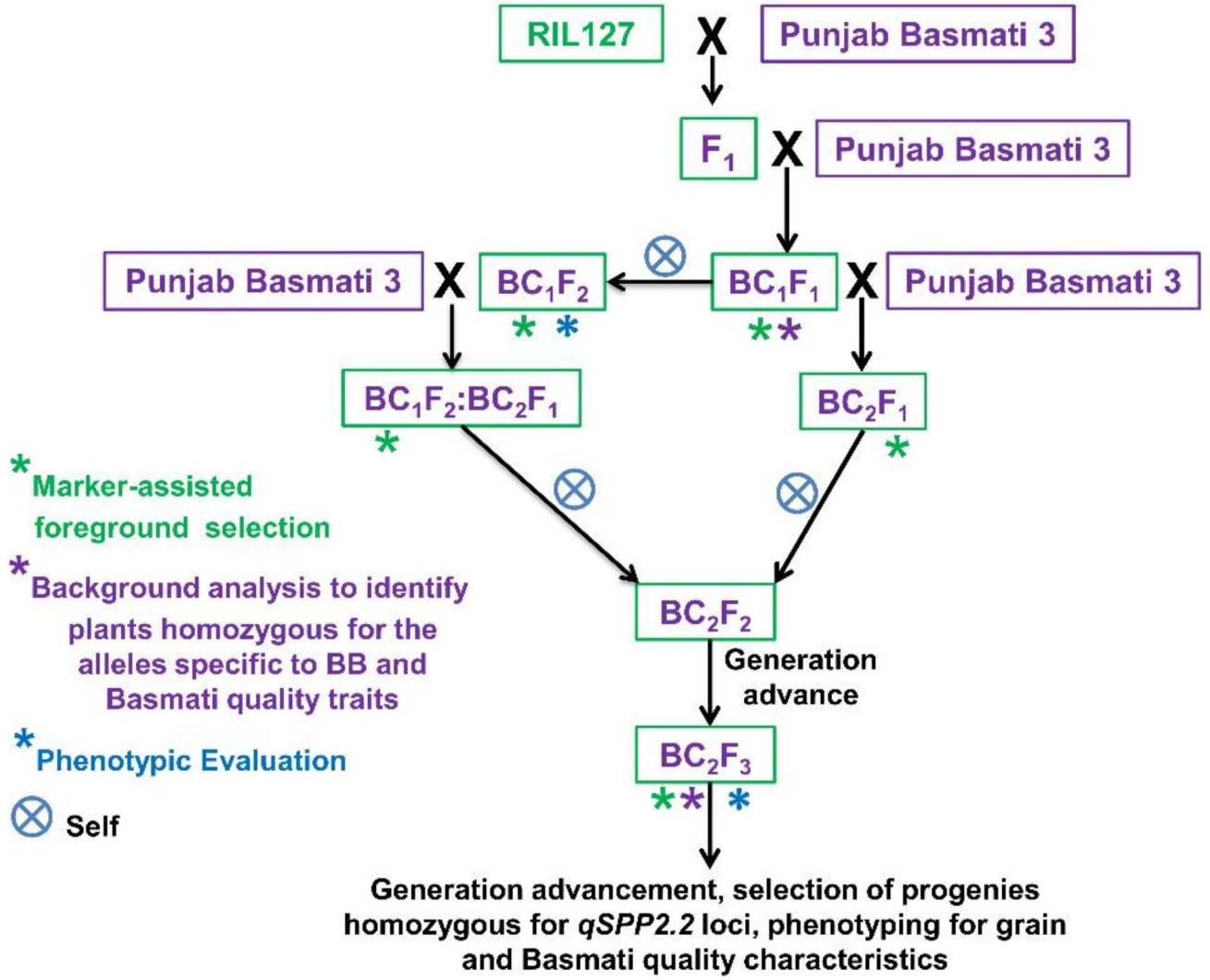

Fig. 1 The overall breeding strategy followed in the study to introgress the QTL, *qSPP2.2* into the recipient parent PB3; RIL127 is the introgression line which carries the QTL; F_1, BC_1F_1, BC_1F_2, BC_2F_1, BC_1F_2:BC_2F_1, BC_2F_2, BC_2F_3 are the various progenies generated from the two parental lines, namely PB3 and RIL127

concentration of 200ng/µl by adding adequate quantity of 1X TE buffer. In vitro PCR amplification was performed in 96-well PCR plate in Thermal Cycler in a 10 µl reaction as per the following temperature profile: initial denaturation at 94ºC for 4 min, denaturation at 94º C for 1 min, annealing at 55–60ºC for 1 min, elongation at 72ºC for 1 min, and final extension at 72ºC for 7 min. The PCR amplified products were resolved using 2.5% agarose gel and visualized under gel documentation system (UVP Imaging).

Marker-Assisted Foreground Selection and Background Analysis for Important Basmati Quality Traits and BB Resistance

For foreground selection, a total of 38 SSR markers spanning the *qSPP2.2* region were identified. Twenty-eight of them were selected from the Project IRGSC (2005) and Kaur et al. 2018. We further designed 10 SSR markers from four BAC clones harboring this QTL (Kaur et al. 2018). The details of primer sequences were provided in Table S1 & S2. These markers were tested for parental polymorphism between RIL127 and PB3. Selected polymorphic markers linked to *qSPP2.2* were used for foreground selection in BC_1F_1 and advanced backcross/self progenies. The selected plants from BC_1F_1 and advanced backcross/self progenies carrying the *qSPP2.2* allele were analyzed with the important background markers for ensuring Basmati quality characteristics, BB resistance etc. A total of 27 SSR markers linked to BB resistance (*Xa21*, *xa13)*, Basmati aroma (*fgr*), intermediate amylose content (RM190 linked to *wx* locus) etc. in the recipient parent PB3 were analyzed (Table S3 & S4) and selected polymorphic markers were used in

background analysis for recovering the BB resistance, Basmati specific traits and other quality characteristics in the backcross progenies. The details of selected polymorphic markers used for foreground selection and background analysis for the quality traits and BB resistance are given in the Table 1.

Phenotypic Expression Analysis and Identification of Superior Plants

To see the effect of *qSPP2.2* in the background of Basmati rice and to identify superior plants, phenotypic data were recorded on parental lines RIL127 and PB3 and their progenies such as BC_1F_2, BC_2F_3 and subsequent advanced selfed progenies during the cropping seasons 2015–2019 at PAU, Ludhiana, India. Data for the agronomic and yield component traits viz., DF, PH, TPP, SPP, SGP, FGP, GL, GB, L/B ratio and grain yield were recorded in the same way as mentioned above. Data were analyzed using Microsoft Excel and GraphPad Prism (v8.0.1).

Results

Genetic Correlation Between GPP and Grain Size in Basmati Rice

Before undertaking transfer of the high grain number conferring allele of the QTL *qSPP2.2*, we studied the correlations using replicated F_2 population generated from the cross PB3 x RIL127. Data for various yield component traits were recorded on 266 individual plants in each replication (Fig. S1 A-D). Mean values of the yield parameters from two replications were used for correlation analysis, which revealed a non-significant correlation between SPP and GL. However, significant but weak negative correlation was observed between SPP and GB (r = -0.150*), and DF (r = -0.170*) (Fig. 2). SPP shows a strong significant positive correlation (0.51***) with GWP. SPP also shows significant positive correlation with fertility percentage (r=0.28**). Analysis of variance revealed replication effects to be non-significant (p>=0.05), but significant differences were observed due to genotypic differences for all the yield component traits (Table 2). Data shows nonsignificant (p>=0.05) replication effects on important agronomic traits like SPP and GL, where SPP varies in between 29.83 and 198.5 with a mean value of 106.46 (R_1) and 105.35 (R_2) respectively. In the F_2 population, the SPP of 202 plants was more than the recurrent parent PB3 (Table 2), demonstrating a significant improvement in the SPP. However, there was a decrease in the TPP of the replicated F_2 plants, which may be due to separation of the tillers during replication. The TPP of 233 plants was more than the RIL127, whereas, only 24 plants had TPP higher than the PB3. PPP varied in between 5 and 36, where majority (230) of the plants had PPP higher than the average value of RIL127 and 26 transgressive segregants were observed. GL varies in between 9.11 mm and 13.62 mm with a mean value of 10.88 (R_1) and 10.83 (R_2) respectively, where 206 plants were found to have GL longer than the introgression line (Table 2). GWP varied in between 1.80 g and 99.80 g, with 176 plants had grain weight higher than the recurrent parent PB3, demonstrating a significant improvement in the grain weight/plant in the replicated F_2 progeny (Table 2).

Marker-Assisted Introgression of *qSPP2.2* Loci into PB3

To facilitate marker-assisted introgression of *qSPP2.2* loci, we screened a total of 38 SSR markers flanking the *qSPP2.2*, previously identified by Kaur et al. (2018), for parental polymorphism between RIL127 and PB3. Five of them, namely RM6, RM13769, RM13745, RM13772 and ASSR-10 (OJ1493-H11-2) were found to be polymorphic between both the parents. All the polymorphic markers were co-dominant except RM13769, which was dominant and amplified only the PB3 allele, not the *O. longistaminata*

Table 1 List of selected polymorphic markers employed for the foreground selection and background analysis

Foreground markers				
Markers	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Amp. size (bp)**	**References**
RM13745	cgcgagtgcaaacaaatcaacc	gggtgagcgctactgctaaactgg	125	Kaur et al. 2018
RM13769	ccaccattcggtaatgagatcg	gacgtggtgatagatggttctgg	400	Kaur et al. 2018
RM13772	acggagcaagtggagcttctcg	gcggcagccatatctctcttgc	326	Kaur et al. 2018
RM6	gtcccctccacccaattc	tcgtctactgttggctgcac	445	Kaur et al. 2018
A-SSR-10	atataaatacacgcgggcgg	gcccgcgtgtatttatattaagtc	246	Kaur et al. 2018
Background markers for Basmati specific traits and BB resistance				
Markers/Genes	**Forward primer**	**Reverse primer**	**Traits**	**References**
Badh2-P10 (*fgr*)	ggccaacgatactcagtgag	ccggtcatcagctaacttcc	Basmati aroma	Temnykh et al. (2000); Jain et al. (2006)
xa13	tcccagaaagctactacagc	gcagactccagtttgacttc	BB resistance	Huang et al. (1997)
Xa21	agacgcggaagggtggttcccgga	agacgcggtaatcgaagatgaaa	BB resistance	Huang et al. (1997)
RM190 (*wx*)	ctttgtctatctcaagacac	ttgcagatgttcttcctgatg	Intermediate Amylose	Temnykh et al. (2000); Akagi et al. (1996)

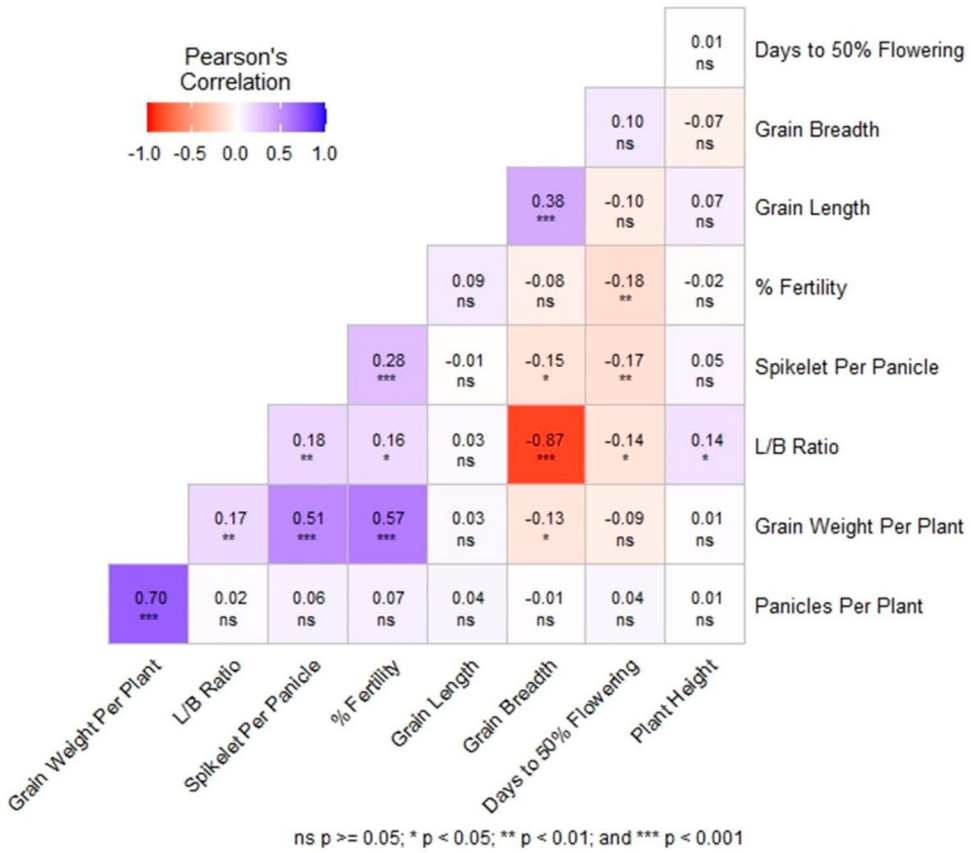

Fig. 2 Correlation among various agronomic and yield traits recorded on replicated F_2 of the cross PB3X RIL127; correlation was absent between SPP and GL (ns, p >= 0.05), however, a significant negative correlation (-0.150*) was observed between SPP and GB at $p < 0.05$

Table 2 Phenotypic data for agronomic and yield traits recorded on replicated F_2 of the cross PB3 X RIL127

Trait	Mean values for PB3	Mean values for RIL127	Population Range	Mean Square	No. of plants showing values > PB3	No. of plants showing values > RIL127
DF*	123.8	116	103–152	263.73	167	219
PH (cm)	97	93.2	87.5–157	514.47	250	256
TPP	19.6	7.8	5-37.5	57.1	24	**233**
PPP	19.4	7.5	5–36	58.2	26	230
SPP	**78.47**	**198.8**	29.83–198.5	1735.17	**202**	1
SF (%)	88.02	78.79	4.59–92.83	938.86	73	96
GL	11.94	10.12	9.11–13.62	1.36	24	**206**
GB	1.85	1.64	0.72–2.95	0.2	55	118
L/B	**6.45**	6.17	4.39–16.08	4.42	**145**	172.5
GWP (g)	17.77	30.78	1.80–99.80	946.11	176	55

*DF- days to 50% flowering; PH- plant height; TPP- number of tillers per plant; PPP- number of panicles per plant; SPP- spikelet per panicle; SF- spikelet fertility; GL- grain length; GB- grain breadth; L/B- L/B ratio; GWP- grain weight per plant

allele (Fig. S2). Among the polymorphic markers, two co-dominant markers, namely RM13745 and RM6 were used for foreground selection in backcross progenies to identify individuals carrying the *qSPP2.2* loci. Additional polymorphic markers between RIL127 and PB3 for Basmati specific aroma (BADH1-Pa, Badh2-P10), BB resistance (*xa13*, *Xa21*), intermediate amylose content (RM190), etc. were also identified for the background analysis of the backcross progenies (Fig. S2).

To introgress the *qSPP2.2* loci, F_1 plants were generated by crossing the RIL127 with PB3 at PAU, Ludhiana, in the year 2014 (Fig. 3A). From these, 10 F_1 plants heterozygous for *qSPP2.2* locus were selected and backcrossed to PB3, producing 2640 BC_1F_1 seeds (Table S5). For rapid generation advancement, the BC_1F_1 plants were handled in two ways. Firstly, around 500 BC_1F_1 plants were planted at NRRI, Cuttack, Odisha, India during the off-season in the year 2014. Of these only 60 plants survived due to

poor germination and low crop stand. Among them, 31 plants were identified as heterozygous and positive for QTL *qSPP2.2* (Fig. 4A and B). The heterozygous plants were further analyzed for the markers linked to Basmati aroma, BB resistance and amylose content (Fig. 4C - F; Table 3). Notably, three lines (Plant IDs. 751−40, 751−50 and 751−54) showed good recovery of recipient parent's alleles for BB resistance, aroma and amylose content.

Backcrossing of the selected BC_1F_1 lines during winter season at NRRI, Cuttack was difficult because of their poor growth and low tillering owing to high photoperiod sensitivity. All the BC_1F_1 heterozygous plants were allowed to self-fertilized, producing enough of BC_1F_2 seeds. The BC_1F_2 seeds from three selected lines were grown as BC_1F_2 population at PAU, Ludhiana, during 2015 Kharif crop season (Fig. 3B), and plants were subjected to marker-assisted foreground selection and phenotyping. A total of 270 BC_1F_2 plants were analyzed using the same polymorphic foreground markers. Among them, 68 plants were homozygous for the donor allele, 130 plants were heterozygous and 72 plants were homozygous for the PB3 allele, showing a segregation ratio of 1:2:1 (Table S6 and S7). All the homozygous plants were self-fertilized and their advanced self-progenies were grown during the 2016–2018 crop seasons at PAU, Ludhiana and plants were evaluated for phenotypic performance and Basmati quality characteristics.

Secondly, during the 2015 Kharif crop season, as many as 1400 BC_1F_1 plants were planted at PAU, Ludhiana (Fig. S3- A & B). Among them, 760 plants were analyzed with polymorphic markers linked to the QTL *qSPP2.2* and 371 plants were identified as heterozygous and positive for QTL *qSPP2.2*. The BC_1F_1 plants heterozygous for the donor parent allele were further analyzed using markers *xa13* and *Xa21*, *Badh2-P10* and RM190. Among the 371 positive plants, 8 plants were identified as heterozygous for *qSPP2.2* and also homozygous at all the recipient parent's alleles

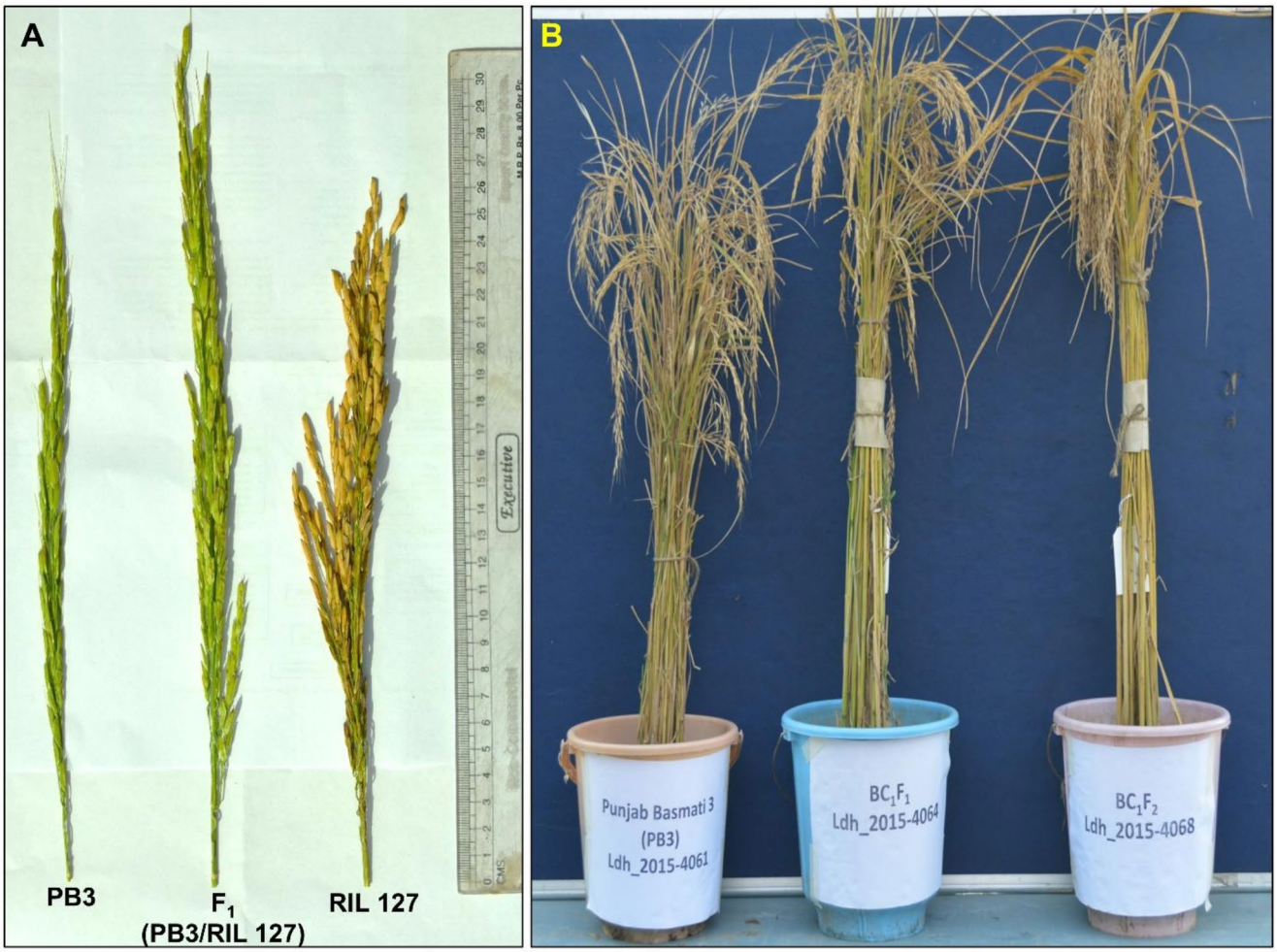

Fig. 3 Panicle and plant morphology of the parental lines PB3 and RIL127, F_1 and their backcross/self progenies. **A**- Panicle size of PB3, RIL127 and their F_1; **B**- Plant type and morphological features of the elite Basmati variety PB3 and the backcross progenies of the cross PB3/RIL127/PB3; PB3 shows thin stem and 20–30 TPP with 65–70 SPP, whereas, the backcross/self progenies namely BC_1F_1, BC_1F_2 had 10–20 TPP with 70–140 SPP (plants from 2015 Kharif season at PAU, Ludhiana)

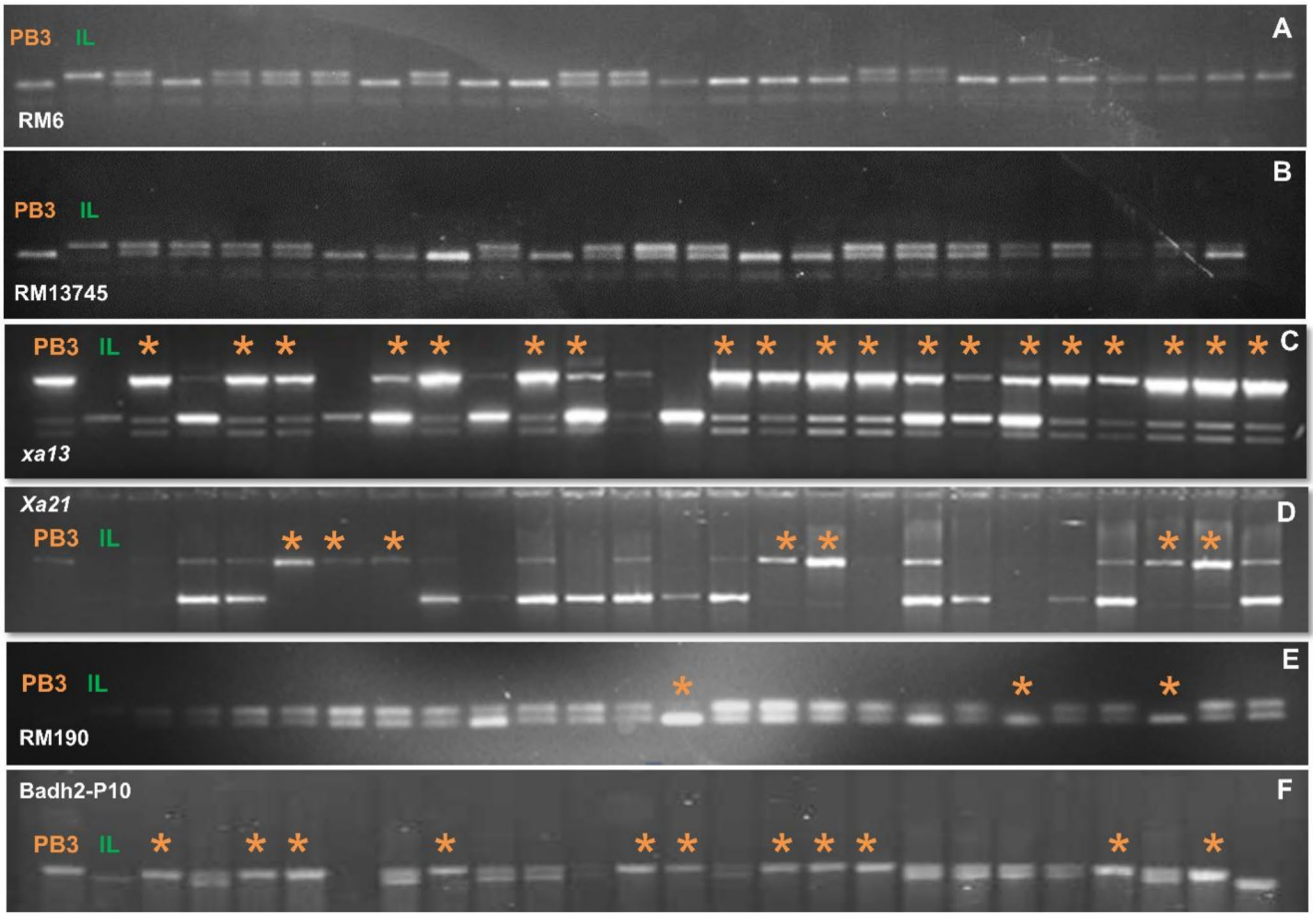

Fig. 4 Marker-assisted foreground selection of BC_1F_1 plants and their background analysis for selected polymorphic markers. **A & B**- BC_1F_1 plants heterozygous at *qSPP2.2* allele were selected based on the polymorphic foreground markers RM6 and RM13745; All the heterozygous plants were subjected to marker-assisted background selection to identify homozygotes for the selected background markers; **C**, **D**, **E & F**- BC_1F_1 heterozygous plants with homozygous/heterozygous alleles of *xa13*, *Xa21*, RM190, and Badh2-P10 (indicated with asterisks), respectively from PB3 were selected

(*xa13*/*Xa21*, Badh2-P10 and RM190) for BB resistance, aroma and intermediate amylose content respectively (Table S8) and 35 heterozygous plants were identified as homozygous for at least three among the four desirable recipient parent's alleles. Hence, from a total of 43 selected plants, 30 plants were backcrossed to the recurrent parent PB3 to generate 3280 BC_2F_1 seeds (Table S9). Nearly 1000 BC_2F_1 plants were planted at PAU, Ludhiana, during the 2016 Kharif crop season and 696 BC_2F_1 plants were analyzed for the *qSPP2.2* loci using the same polymorphic foreground markers (Fig. S4 A-B; Table S10) and positive plants were identified and self-fertilized to produce BC_2F_2 progeny. A substantially large number of BC_2F_2 plants were grown during the 2017 Kharif crop season at PAU, Ludhiana and self-fertilization was done for generation advancement. During the next Kharif crop season, BC_2F_3 plants were subjected to marker assisted foreground selection for the *qSPP2.2* loci and background analysis for BB resistance, aroma and amylose content. The plants homozygous for the said alleles were identified and self-fertilized for generation advancement and identification of homozygous line showing increased GPP.

Identification of Superior Plants in BC_1F_2, and BC_2F_3 Progenies

Phenotypic data were recorded on parental lines PB3 and RIL127 and three selected BC_1F_2 lines (Fig. 5A and B). Thirty phenotypically superior (thick stem, moderate tiller number and plant height) BC_1F_2 plants heterozygous for the *qSPP2.2* allele with higher SPP (more than 160) were backcrossed to the recurrent parent PB3 to generate 1993 BC_1F_2:BC_2F_1 seeds (Table S11). The seeds were raised during the 2016 Kharif crop season at PAU, Ludhiana, and self-fertilization was done for selected plants to obtain BC_2F_2 progeny. After a generation advancement, the BC_2F_3 plants were grown at PAU during 2018 crop season and a substantially large number of desirable homozygous plants were

Table 3 Foreground selection and background analysis of the BC_1F_1 plants of the cross PB3/RIL127/PB3

Plant ID	*qSPP2.2* allele	Badh2-P10 allele	*Xa21* allele	*xa13* allele	RM 190 allele
PB3	—	+	+	+	+
RIL127	+	—	—	—	—
751-3[a]	1	+	1	1	1
751-4	1	1	1	1	1
751-6	1	+	1	1	1
751-7	1	+	+	1	1
751-8	1	NA	+	—	1
751-9	1	1	+	1	1
751−11	1	+	1	1	1
751−12	1	1	—	1	1
751−13	1	1	1	1	1
751−14	1	1	—	1	1
751−18	1	1	1	1	1
751−22	1	+	—	—	+
751−24	1	1	1	1	1
751−25	1	1	+	1	1
751−26	1	1	+	1	1
751−27	1	1	+	1	1
751−28	1	1	1	1	1
751−30	1	1	—	1	1
751−32	1	1	+	1	+
751−33	1	1	1	1	1
751−34	1	+	1	1	1
751−35	1	1	+	1	+
751−40	1	+	+	1	1
751−42	1	—	1	1	1
751−44	1	1	—	1	1
751−50	1	+	+	—	+
751−51	1	+	+	1	1
751−52	1	1	—	1	1
751−53	1	+	—	NA	1
751−54	1	+	+	1	+
751−56	1	+	—	1	+

[a] 751 is the plant ID of the BC_1F_1 of the cross PB3/RIL127/PB3; + indicates presence of the desirable allele; — indicates absence of the desirable allele; 1 indicates heterozygote; NA means not amplified

identified (Fig. S4 C) and data were recorded on yield component and agronomic traits.

Recurrent parent PB3 showed TPP of 20–25, DF of 110–120 days, PH of 95–110 cm, SPP of 60–80, spikelet fertility of 75–87% and GL of 11–12 mm. Phenotypic and yield component data of 84 plants from the best-selected BC_1F_2 line and 68 plants from the best-selected BC_2F_3 line are presented below: TPP varied from 4 to 30, where largely plants showed significantly higher TPP than the RIL127 (7–8); PH varied from 93 to 155 cm, where majority of the plants showed PH higher than both the parents; SPP varied from 44.6 to 198.8 and majority of the plants had 70–120 SPP, demonstrating a 30–40% increase in the SPP of the recurrent parent PB3 (60–80 SPP); grain L/B ratio varied from 4.0 to 11.54, where mostly plants had typical slender Basmati-type grain with L/B>4.5; the grain yield of majority plants falls within the range of 25–35 q/acre and the majority plants showed significantly higher grain yield than the recurrent parent PB3 (16–17 q/acre), The average yields observed in BC_1F_2 and BC_2F_3 were 24.90 and 22.26 q/acre; DF varies from 99 to 139; spikelet fertility varies from as low as 10.2% to as high as 93.4%; GL and GB varied from 8.93 to 17.70 mm and 0.86–3.44 mm, respectively (Fig. 6A-J & Fig. S5 A-H; Table S12 & Table S13).

Data analysis shows a 35–40% increase in GPP, demonstrating a significant improvement in the grain yield of the plants. Thus, transgressive segregants were observed for most of the yield component traits and a prominent and clear phenotypic effect of *qSPP2.2* loci on the BC_1F_2 and BC_2F_3 populations were observed. The advanced progenies were maintained and evaluated for the important yield component traits during the subsequent crop seasons at PAU, Ludhiana.

Discussion

Correlation Between GPP and Grain Size in Basmati Rice

The traditional and evolved Basmati rice cultivars including PB3, in general, have long grains but with lower GPP compared to modern day cultivars of other rice types and they are low yielding (Singh et al. 2000, 2014; Bhatia et al. 2011). Examining the potential negative correlation between grain number and grain length in Basmati rice is critical if we aim to enhance its productivity at par with the *Indica* rice cultivars. Therefore, prior to attempting transfer of *qSPP2.2* into PB3, one of our objectives was to understand if there is any negative correlation between GPP and the grain size. The F_2 replicated data analysis revealed non-significant replication effects with no significant correlations between the total SPP and GL which is unlike of Fang et al. (2016) and Zahid et al. (2006), where the latter group shows a significant negative correlation (-0.2195) between GL and GPP. This suggests that SPP is independently governed trait in Basmati rice and it does not exhibit pleotropic effects. Also indicates that the grain number QTL *qSPP2.2* can be transferred into PB3 for improving SPP without disturbing the GL, which is the most important trait for Basmati consumption and trading. However, significant but weak negative correlation (-0.150*) was observed between total SPP and GB, which indicates the potential of transferring *qSPP2.2* into Basmati varieties for improving productivity per se and Basmati rice production could be increased without disturbing its grain quality traits. The study is a new report, as the

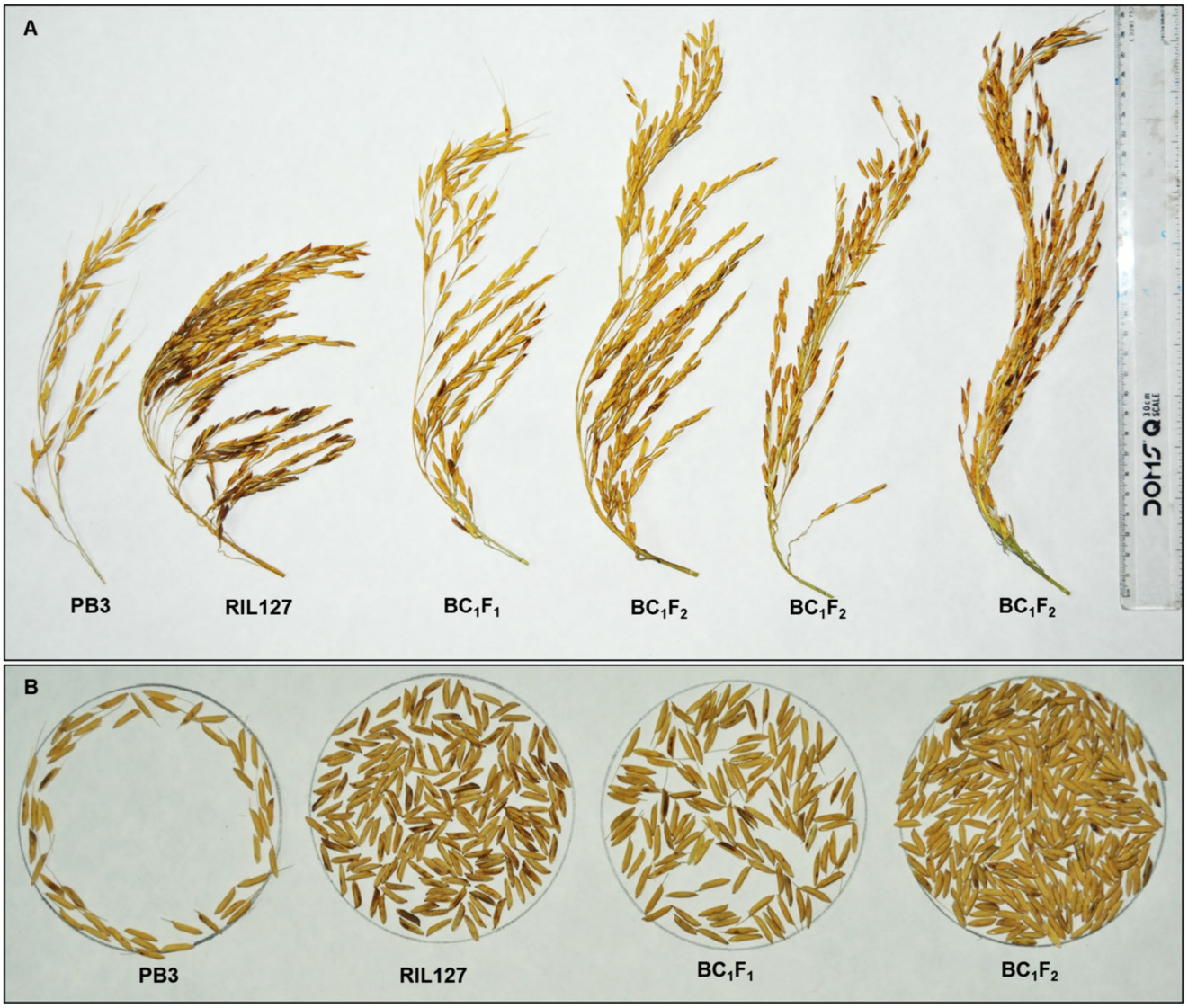

Fig. 5 Variation in panicle size and SPP in the parental lines PB3 and RIL127 and their progenies. **A**- PB3 has comparatively shorter (10–12 cm) panicle with few primary branches than RIL127 and their backcross/self progenies, which have larger panicles (20–25 cm) with increased number of primary and secondary branches; **B**- Number of SPP in PB3 (60–80) was significantly lower than the SPP in RIL127 (160–170) and backcross/self progenies (120–170), each circle contains grains from a single panicle from the respective plants

correlation between GPP and grain size in Basmati rice has not yet been studied adequately.

Introgression of *qSPP2.2* Loci into PB3 Through Marker-Assisted Backcrossing (MABC)

The true hybrid nature of the F_1 plants was determined by analyzing them using mainly two polymorphic co-dominant markers (RM13745 and RM6) linked to the *qSPP2.2* loci (Kaur et al. 2018). The marker-assisted foreground selection of the backcross/advanced progenies was done by employing these two polymorphic markers. Among the 23 aroma-specific markers linked to *BADH1* and *Badh2* genes, only two markers, namely BADH1-Pa and Badh2-P10, could actually differentiate between alleles from RIL127 and PB3. Since both fragrant and non-fragrant rice possess the *BADH1* gene, the recovery of the most important trait of Basmati rice (unique aroma due to the accumulation of 2-acetyl-1-pyrroline) in all the backcross/self progenies was done by using mainly the *Badh2-P10* marker (Lorieux et al. 1996; Temnykh et al. 2000; Jain et al. 2006; Chen et al. 2006; Fitzgerald et al. 2008; Hashemi et al. 2013). PB3 was found to be resistant to all the 10 prevalent pathotypes of *Xanthomonas oryzae* pv. *oryzae* in the state of Punjab (Bhatia et al. 2011; Singh et al. 2014). Hence, the BB resistance capability of the backcross progenies was determined by using mainly two polymorphic markers linked to the recessive gene *xa13* and the dominant gene *Xa21* (Khush et al.

1989, 1990; Ikeda et al. 1991; Zhang et al. 1996; Huang et al. 1997; Sanchez et al. 1999; Rajpurohit et al. 2010; Pandey et al. 2013; Pradhan et al. 2015; Arunakumari et al. 2016). The intermediate amylose content of the Basmati rice was confirmed by analyzing the allelic status of the *wx* loci linked to the polymorphic marker RM190 (Akagi et al. 1996; Temnykh et al. 2000; Rajpurohit et al. 2010; Kottearachchi et al. 2014).

The plants for the targeted QTL *qSPP2.2* were selected in each generation based on the criteria that the foreground markers must be in heterozygous condition in BC_1F_1, BC_1F_2:BC_2F_1 and BC_2F_1 generations while in homozygous condition in BC_1F_2, BC_2F_2, BC_2F_3 and their advanced self-progenies (Bhukya et al. 2020). Thus, the larger BC_1F_1 and BC_2F_1population were raised to ensure identification of a few plants having donor parent allele viz., heterozygous positive for *qSPP2.2* and homozygous at most all the markers required for background analysis to ensure BB resistance, Basmati aroma and intermediate amylose content. In the BC_1F_1, 50% of the plants were expected to have the donor parent allele (*qSPP2.2*) in heterozygous condition and the remaining 50% homozygous for the recipient parent allele (Olalekan et al. 2019; Chukwu et al. 2020). In the current study, among the 820 BC_1F_1 plants analyzed, 402 plants (49.02%) were heterozygous for the donor parent allele and they were selected for further marker analysis for the recovery of Basmati quality characteristics and BB resistance. Codominance nature of the selected foreground markers linked to the *qSPP2.2* loci helped to identify BC_1F_1 plants heterozygous for the allele for effective selection of plants harboring *qSPP2.2* loci for further backcrossing. This finding is more evident from the non-significant χ^2 value of 0.312, indicating that the observed data fits the expected 1:1 ratio of heterozygous and homozygous plants for *qSPP2.2* in BC_1F_1 at a significance level (α) of 0.05 (Rajpurohit et al. 2010). However, the proportion of heterozygous positive plants for the QTL *qSPP2.2* was decreased in the subsequent backcross generations as evident from the marker-assisted foreground selection of BC_2F_1 and BC_1F_2:BC_2F_1 progenies (Table S10). The above observation could be attributed to a fundamental mechanism that the number of meiotic crossover events is gradually increases as the generation advances (Bhukya et al. 2020). As a result, the linkage between the marker and the QTL breaks, which led to a lower number of heterozygous positive plants in BC_2F_1 progeny. In the BC_1F_2, 25% of the plants were expected to have the *qSPP2.2* allele in homozygous condition, 50% in heterozygous condition and the remaining 25% plants homozygous for the recipient parent allele (Pandey et al. 2013; Olalekan et al. 2019; Chukwu et al. 2020). From a total of 270 BC_1F_2 plants analyzed, 68 plants were homozygous for the donor allele, 130 were heterozygous and 72 plants were homozygous for the PB3 allele (Table S6 and S7), showing a non-significant χ^2 value of 0.489, indicating that the observed segregation fits the expected 1:2:1 ratio of the *qSPP2.2* loci in BC_1F_2 progeny at a significance level (α) of 0.05.

The plants retaining some of the highly important PB3 characteristics were selected in each generation based on the criteria that the background marker alleles for BB resistance, Basmati aroma and intermediate amylose content must be in homozygous condition (Rajpurohit et al. 2010; Pandey et al. 2013; Singh et al. 2023). As comprehensive background selection for large numbers of plants with markers distributed throughout the genome at each backcross generation is an expensive strategy, marker-assisted foreground selection combined with background marker analysis to ensure retention of some of the highly important PB3 characteristics, such as BB resistance, Basmati aroma and intermediate amylose content, and tracking recovery of the RP genome in early generations based on phenotype and assessment of background genome recovery in advanced generations through comprehensive marker analysis/sequencing of the complete genome was adopted here as a rapid and efficient strategy to introgress the QTL *qSPP2.2* into PB3 (Pandey et al. 2013). In the current study, from a total of 402 BC_1F_1 heterozygous positive plants, 9 plants were identified as homozygous at all the recipient parent's alleles for BB resistance (*xa13*/*Xa21*), aroma (Badh2-P10), and intermediate amylose content (RM190) and 37 heterozygous plants were identified as homozygous for at least three among the four desirable recipient parent's alleles (Table 3 and Table S8). This observation demonstrates that the number of BC_1F_1 plants with all four desirable alleles for recovering the critical PB3 quality traits is less than the plants possessing either three or two desirable alleles in the backcross progeny and the observation was found to be similar with the previously reported studies by Rajpurohit et al. 2010; Bhatia et al. 2017; Bhukya et al. 2020 and Singh et al. 2023. However, in BC_2F_3 progenies, homozygosity for the *xa13*/*Xa21*, Badh2-P10 and RM190 alleles increased substantially as a result of backcrossing and repeated selfing, which systematically introduced more of the recipient parent's genetic material into the progeny. Hence, the proportion of homozygosity for these alleles was significantly higher in BC_2F_3 progenies due to the cumulative effect of selection and segregation. The larger BC_1F_1, and BC_2F_3 populations were generated to ensure retention of important Basmati quality traits, BB resistance of PB3 along with the target trait of higher SPP and important grain quality features and recovery of the PB3 genome in their advanced progenies in a rapid and efficient molecular breeding strategy to develop a high-yielding improved PB3.

The Transferred QTL *qSPP2.2* from *O. longistaminata* Improves Grain Number in Basmati Rice

Phenotyping and grain number analysis of the BC_1F_2 and BC_2F_3 plants from the selected lines harboring *qSPP2.2*

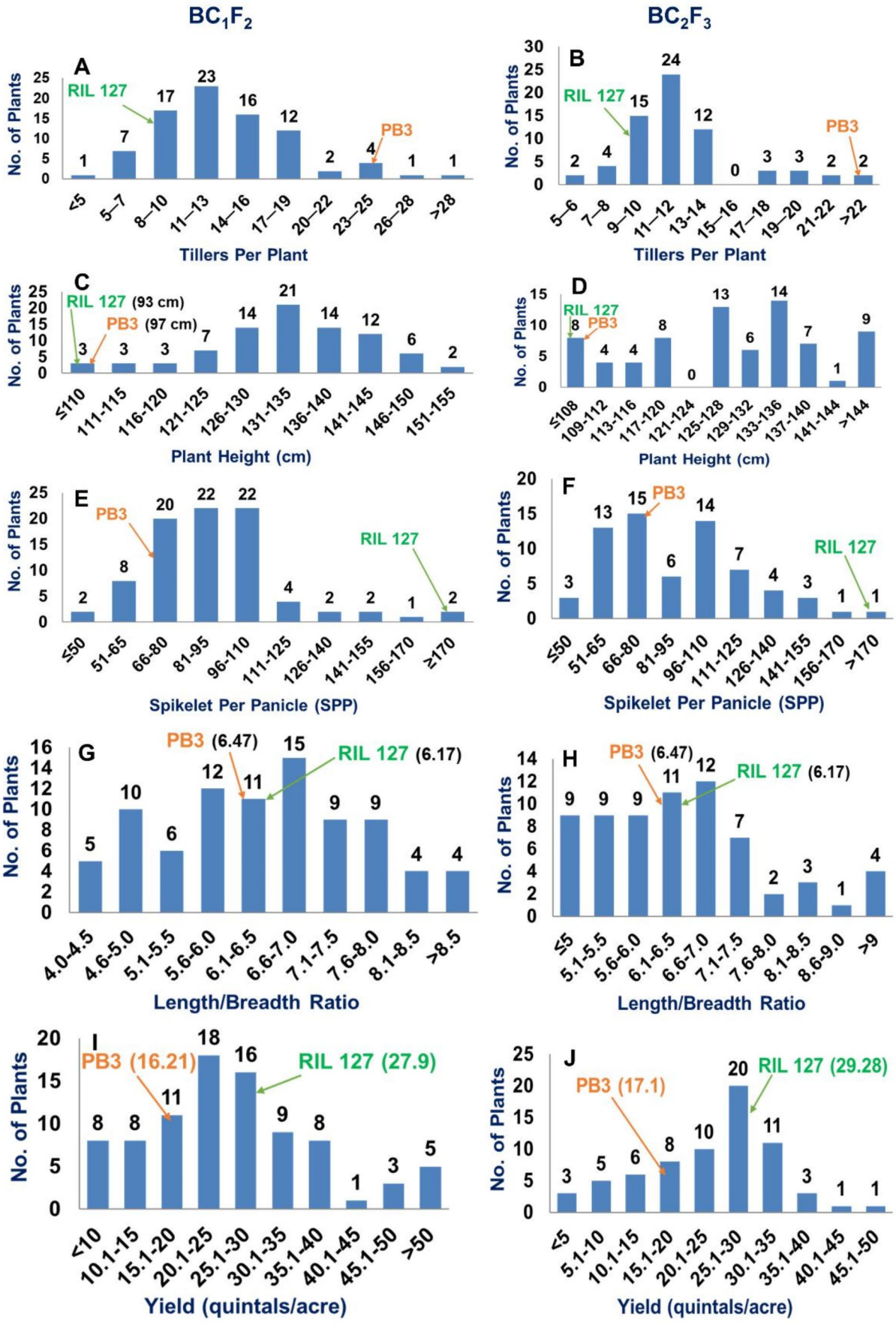
BC1F2
BC2F3
A
No. of Plants
RIL 127
PB3
1 7 17 23 16 12 2 4 1 1
<5 5–7 8–10 11–13 14–16 17–19 20–22 23–25 26–28 >28
Tillers Per Plant
B
No. of Plants
RIL 127
PB3
2 4 15 24 12 0 3 3 2 2
5–6 7–8 9–10 11–12 13-14 15–16 17–18 19–20 21-22 >22
Tillers Per Plant
C
No. of Plants
RIL 127 (93 cm)
PB3 (97 cm)
3 3 3 7 14 21 14 12 6 2
≤110 111-115 116-120 121-125 126-130 131-135 136-140 141-145 146-150 151-155
Plant Height (cm)
D
No. of Plants
RIL 127
PB3
8 4 4 8 0 13 6 14 7 1 9
≤108 109-112 113-116 117-120 121-124 125-128 129-132 133-136 137-140 141-144 >144
Plant Height (cm)
E
No. of Plants
PB3
RIL 127
2 8 20 22 22 4 2 2 1 2
≤50 51-65 66-80 81-95 96-110 111-125 126-140 141-155 156-170 ≥170
Spikelet Per Panicle (SPP)
F
No. of Plants
PB3
RIL 127
3 13 15 6 14 7 4 3 1 1
≤50 51-65 66-80 81-95 96-110 111-125 126-140 141-155 156-170 >170
Spikelet Per Panicle (SPP)
G
No. of Plants
PB3 (6.47)
RIL 127 (6.17)
5 10 6 12 11 15 9 9 4 4
4.0-4.5 4.6-5.0 5.1-5.5 5.6-6.0 6.1-6.5 6.6-7.0 7.1-7.5 7.6-8.0 8.1-8.5 >8.5
Length/Breadth Ratio
H
No. of Plants
PB3 (6.47)
RIL 127 (6.17)
9 9 9 11 12 7 2 3 1 4
≤5 5.1-5.5 5.6-6.0 6.1-6.5 6.6-7.0 7.1-7.5 7.6-8.0 8.1-8.5 8.6-9.0 >9
Length/Breadth Ratio
I
No. of Plants
PB3 (16.21)
RIL 127 (27.9)
8 8 11 18 16 9 8 1 3 5
<10 10.1-15 15.1-20 20.1-25 25.1-30 30.1-35 35.1-40 40.1-45 45.1-50 >50
Yield (quintals/acre)
J
No. of Plants
PB3 (17.1)
RIL 127 (29.28)
3 5 6 8 10 20 11 3 1 1
<5 5.1-10 10.1-15 15.1-20 20.1-25 25.1-30 30.1-35 35.1-40 40.1-45 45.1-50
Yield (quintals/acre)

Fig. 6 Phenotypic expression of *qSPP2.2* in the BC_1F_2 and BC_2F_3 plants. **A&B**- Tillers Per Plant, majority of the plants showed 10–15 TPP, which were comparatively higher than the RIL127 (7–8), but lower than PB3 (~20); **C&D**- Plant Height, majority of the plants showed PH higher than their parents; **E&F**- Spikelet Per Panicle, largely plants showed 70–120 SPP, which were nearly 30–40% higher than the recipient parent PB3 (60–80 SPP); **G&H**- L/B Ratio, mostly plants had typical slender Basmati-type grain (L/B>4.5); **I&J**- Grain Yield, largely plants showed significantly higher grain yield than the recurrent parent PB3 (16–17 q/acre) with majority falling in the range of 25–35 q/acre, the average yields observed in BC_1F_2 and BC_2F_3 were 24.90 and 22.26 q/acre, respectively; arrows indicate the positions of recurrent parent PB3 and introgression line RIL127

allele from the donor parent and other desirable alleles for BB resistance, Basmati aroma and intermediate amylose content showed significant improvement in grain numbers along with the longer and finer grains, which are the important phenotypic characteristics of Basmati rice (Siwach et al. 2004; Singh et al. 2018). Moreover, transgressive segregants were obtained for the majority of the yield component parameters, including SPP and grain yield, where a significant improvement in the SPP of BC_1F_2 and BC_2F_3 was observed and a 35–40% increase in grain number was recorded. PH of PB3 was somewhat longer than the RIL127, and their progenies also show longer height than the parents. However, if we compare PB3 with its parent variety, the height of PB3 was shorter (105 cm) than its parent variety Basmati 386 (158 cm). Moreover, due to thick stem, the lodging score of PB3 was comparatively lower (1) than Basmati 386 (9) and another popular check varieties of Basmati rice, PB1121 (3) (Singh et al. 2014). Therefore, PB3 was considered as semi-dwarf and lodging tolerant variety and the progenies generated in this study also show thick stem and lodging resistance.

These results were found to be similar to the study reported by Bhukya et al. 2020; where marker-assisted introgression of yield QTLs into elite rice varieties showed a yield advantage of 32–84% under moisture stress. These observations were also found similar to Khan et al. (2009), which supports our findings of significant improvement in grain numbers in BC_1F_2 and BC_2F_3 progenies. The present study indicated that the traditional and evolved Basmati rice cultivars are low yielding largely due to the lower number of SPP, which is the result of unavailability of superior grain number increasing alleles in Basmati rice. Yield potential of elite Basmati varieties could be improved by increasing the SPP through transferring the spikelet or grain number QTLs or genes governing grain number in rice from wild relatives or landraces into low yielding Basmati varieties. The identified QTL *qSPP2.2*, which confers higher SPP from *O. longistaminata* was found to be able to increase the grain number by at least>35% in PB3. Upon introgression of *qSPP2.2* loci into PB3, the allele, which confers higher grain number leads to an increase in SPP (Kaur et al. 2018). Resulting in the significant improvement of grain yield of the introgressed lines. These results were found to be consistent with the reports by Kaur et al. (2018).

The correlation coefficients among various yield-contributing traits, including SPP and grain size in the F_2 population, large population size in BC_1F_1, BC_2F_1 and BC_2F_3, and their marker-assisted foreground selection and background analysis for important Basmati quality traits and BB resistance and observed phenotypic characteristics in BC_1F_2, BC_2F_3 did indicate that, following MAS, it should be possible to develop high-yielding Basmati varieties that could benefit both farmers and consumers alike. This approach may offer a viable strategy to bridge the productivity gap between traditional Basmati varieties and modern high-yielding *Indica* rice cultivars, without compromising the unique quality traits of Basmati rice after a comprehensive background recovery analysis and evaluating all phenotypic data on critical Basmati-specific traits such as aroma, alkali spreading value, amylose content, chalkiness, and gel consistency, which have already been initiated by the same research group.

Conclusions

The introgression of *qSPP2.2* locus into the genetic background of the PB3 resulted in a notable increase in SPP, which accounts for 35–40% of the observed phenotypic variability in grain number, demonstrating the potential of utilizing the locus in rice breeding programs for enhancing productivity in Basmati and cultivated rice varieties. Correlation analysis between SPP and grain length reveals no significant correlation, suggesting that these traits are largely independent. This study indicates that the grain number in Basmati rice could be increased without impacting the long and slender grain characteristic. However, a weak negative correlation (-0.150*) between SPP and grain breadth, implying that as the number of SPP increases, there may be a slight reduction in grain breadth. This trade-off may be critical for maintaining grain quality and overall grain appearance, which are important characteristics in Basmati rice. The introgressed lines exhibited superior phenotypic traits, including longer and finer grains, resistance to BB (via *xa13* and *Xa21* markers), good aroma (tracked by Badh2-P10 markers), and intermediate amylose content (tracked by RM190). These traits make the improved lines suitable for both farmers seeking higher yields and consumers prioritizing grain quality. In conclusion, we are very near to an improved version of PB3 with significantly higher SPP, which has been achieved through marker-assisted introgression of *qSPP2.2* loci from *Oryza longistaminata* into Punjab Basmati 3.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12042-025-09406-3.

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Author Contributions **S.D**: writing—original draft, conceptualization, methodology, investigation, data curation, formal analysis, software; **K.N** and **D.B**: conceptualization, methodology, supervision and Writing- reviewing and editing; **A.K** and **K.K**: methodology, visualization, data curation, Writing- reviewing and editing; **K.S**: conceptualization, resources, methodology, supervision, visualization, data curation, Writing- reviewing and editing. All the authors have read and edited the draft manuscript and agreed to the final manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethics Approval Not applicable.

Competing Interests The authors declare no competing interests.

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