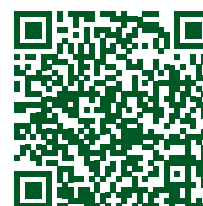


Genetic improvement of Chakhao rice by gene stacking of high-yielding and durable blast-resistant traits

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Crop Breeding and Applied Biotechnology
24(3): e47772437, 2024
Brazilian Society of Plant Breeding.
Printed in Brazil
<http://dx.doi.org/10.1590/1984-70332024v24n3a32>



Abstract: *Chakhao is a popular pigmented black rice variety with remarkably high anthocyanin content. Due to its susceptibility to various biotic and abiotic stresses, its production has remained low. Here, two genes, Pi54 blast-resistant and OsSPL14, high-yielding genes of rice, were pyramided in a Chakhao landrace by crossing with CR Dhan 307. From a total of 147 F₄ lines developed, 32 were identified with the positive alleles of both genes, of which 16 were black grain coloured, and their total anthocyanin content ranged from 30.19±2.19 to 240.31±2.62 mg 100 g⁻¹ dried weight. Among these, ChM 68 F₄ line had the highest anthocyanin content (240.31 ± 2.62 mg 100 g⁻¹ of powdered grain) with more 118.96 mg 100 g⁻¹ anthocyanin than the recipient parent, CHK13 (121.35±3.32 mg 100 g⁻¹), making it a promising line to be released as a high-yielding and durable blast-resistant Chakhao variety in Manipur.*


Keywords: *Anthocyanin, Chakhao, manipur black rice, ideal plant architecture, Pi54*

INTRODUCTION

Manipur Black Rice or Chakhao, as it is locally known, is a specialty rice GI tagged to the northeastern state of Manipur, India, known for its fragrant odour and purplish-black colour (Asem et al. 2017). Some of the commonly found black rice landraces in Manipur are Chakhao Poireiton, Chakhao Amubi, Chakhao Pungdol Amubi, Chakhao Sempak, Pong Chakhao, Wairi Chakhao and Khurkhul Chakhao. Some of these Chakhao landraces could be white-grained, but still have the fragrant smell characteristic of black Chakhao. Of the many Chakhao landraces found, Chakhao Poireiton is the popular and most cultivated landrace, accounting for ~ 43% of the total Chakhao cultivation as well as production in Manipur (Asem et al. 2017, Borah et al. 2018). Several delicacies made in Manipur using Chakhao such as cakes, desserts (Chakhao kheer), chapatis (Chakhao tal), beverages, and utong chak (Chakhao cooked in bamboo) are extremely popular (Roy et al. 2014, Asem et al. 2015). The extensive use of Chakhao in religious and festive ceremonies in Manipur has made this particular rice variety indispensable to the state.

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Received: 13 December 2023

Accepted: 02 June 2024

Published: 15 June 2024

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The colour of this pigmented rice variety is due to the presence of the secondary metabolite, anthocyanin. These pigments can scavenge reactive oxygen species and can therefore act as antioxidants. Chakhao is found to contain relatively higher amounts of anthocyanin as compared to other cereal and food products. A study found the anthocyanin content of Chakhao Poireiton to be 740 mg kg⁻¹ while that of Chakhao Amubi was found to be 692 mg kg⁻¹ (Asem et al. 2015). Due to its high anthocyanin content, Chakhao has many nutritional as well as medicinal properties. It has been known to impart many nutraceutical benefits such as the reduction of the risk of cardiovascular diseases and cancer. Consumption of the outer layer of black rice was also successful in inhibiting atherosclerotic plaque formation in rabbits (Ling et al. 2002) and also in apolipoprotein E-deficient (Apo-E) mice (Xia et al. 2003). Due to its many attractive and healthy attributes, Chakhao is currently in huge demand, both in the national and in the international markets. However, its demand has not been met as its cultivation can be quite taxing, owing to its many inherent traits such as low grain yield and high susceptibility to biotic as well as abiotic stresses (Borah et al. 2018).

Rice blast caused by the fungus *Magnaporthe oryzae* of the Ascomycetaceae family is one of the most common and disastrous diseases in rice. Rice blast can affect tissues of the plant above ground level such as leaves, leaf collars, culms, culm nodes, panicles and panicle neck nodes by making lesions on them. About a total of 100 blast-resistant genes have been identified so far, out of which 27 have been characterized. Some of these genes are *Pi9*, *Pita*, *Pi2*, *Pi37*, *Pi54*, *Pi21* etc. (Yadav et al. 2019). The *Pi54* gene is one of the most durable blast-resistant genes that have shown impressive resistance against the disease (Rai et al. 2011). It is one of the few broad-spectrum R-genes that have shown resistance against leaf blasts (Ning et al. 2020). The *Pi54*, formerly known as *Pi-kh*, belongs to the Coiled Coil- Nucleotide Binding Site-Leucine Rich Repeats' (CC-NBS-LRR) class of R-genes which is known to activate several downstream related pathways upon pathogen interaction (Vasudevan et al. 2015). Many studies on the successful introgression of blast R-genes in rice through the use of Marker-Assisted Selection (MAS) have shown effective and durable results against blast disease caused by *M. oryzae* (Hittalmani et al. 2000, Singh et al. 2001, Fukuoka et al. 2015, Tanweer et al. 2015, Xiao et al. 2016, Kumari et al. 2017).

Yield is a complex quantitative trait governed by many QTLs, and different QTLs affecting grain yield have been cloned in rice during the last two decades (Ngangkham et al. 2018). Another gene, *OsSPL14*, is known to promote panicle branching in rice and hence is responsible for the increase in yield (Miura et al. 2010). *OsSPL14* is responsible for regulating the branching of shoots and the number of grains per panicle of rice plants and is therefore even known as the *Wealthy Farmer's Panicle (WFP)* or *Ideal Plant Architecture (IPA)* gene (Jiao et al. 2010).

In this investigation, an improvement of yield and blast-tolerant Chakhao landrace was attempted by pyramiding two genes, *OsSPL14* and *Pi54* genes, followed by a selection of promising black-grained Chakhao varieties with high anthocyanin content through gene-based molecular markers.

MATERIAL AND METHODS

Plant materials

A set of 40 different Chakhao landraces were collected from different parts of Manipur, India (lat 23° 83' N – 25° 68' N, long 93° 03' E – 94° 78' E) and screened for the presence of *Pi54* by genotyping using functional gene-based marker, Pi54MAS. A Chakhao landrace carrying the positive allele of *Pi54* for rice blast resistance with good grain quality was selected for the recipient parent and crossed to introgress *OsSPL14/IPA1* from CR Dhan 307 with a positive allele of *OsSPL14* gene. The F₁s were selfed to develop F₂s and then to F₃s and F₄s. A total of 147 F₄ lines were developed and used for foreground and background selection.

Genomic DNA isolation

Fresh leaves of rice samples were collected separately, frozen in liquid nitrogen, and stored at -80 °C for further use. Genomic DNA isolation was carried out using the CTAB method with slight modification (Doyle and Doyle 1990, Ngangkham et al. 2020). The DNA pellets were dissolved in 50-100 µL⁻¹ 1X TE buffer and the eluted DNA was diluted to get 50 ng µL⁻¹ concentration for PCR amplification.

PCR amplification for genotyping

PCR amplification was carried out with 25 ng template DNA in 10 μ L PCR reaction volumes containing 1X PCR Master mix (Dream Taq Green PCR Master Mix 2X, Thermo Fisher Scientific), with forward and reverse primers. A total of 35 cycles of PCR amplification was carried out using the Mastercycler Nexus Gradient Thermal cycler (Eppendorf, Hamburg, Germany) with the following PCR cycling conditions: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 45 s, extension at 72 °C for 1 min, final extension at 72 °C for 10 min and followed by a holding temperature of 4 °C. The amplified PCR products were separated on 1.5% agarose or 3.5% Metaphor agarose gel (Lonza, USA) electrophoresis system depending on amplicon size and visualized using Gel Doc XR+ Gel Documentation unit (Bio-Rad, Hercules, California, USA). The scored data of the amplified PCR bands were used as genotyping data.

Foreground selection of F_4 lines using functional gene-based markers

The F_4 lines/genotypes were screened using functional gene-based markers of *Pi54* and *OsSPL14* genes. The Pi54MAS for the *Pi54* gene and SPL14-12SNP markers for the *OsSPL14* gene were used as gene-based markers. The Pi54MAS markers with primer sequences (Forward: 5'-CAATCTCCAAAGTTTTCAGG-3' and Reverse: 5'-GCTTCAATCACTGCTAGACC-3') were used with similar PCR cycling and annealing temperature of 55 °C and the PCR products were separated in 1.5% agarose gel. The positive allele of *Pi54* was determined when the markers were amplified at 216 bp against the 359 bp fragment. Similarly, the SPL14-12SNP markers with primer sequences (Forward: 5'-CAAGTGAGACTTCATGTGGT-3' and Reverse: 5'-GTTTCAAGCTTTACGTTGGA-3') were used with similar PCR cycling and annealing temperatures. The positive allele of the *OsSPL14* gene was determined only when the markers were amplified at 302 bp otherwise null for the negative allele.

Background selection of selected/introgressed F_4 lines using SSR markers

For background selection in advanced breeding lines, a polymorphism survey was conducted using the genomic DNA of parental lines. A total of 293 SSR or RM markers covering the whole 12 rice chromosomes were collected from the Gramene Marker database (<http://www.gramene.org/markers/microsat/>). The PCR amplification was carried out as described earlier, and PCR products were separated using a 3.5% Metaphor agarose gel (Lonza, USA) electrophoresis system.

Estimation of anthocyanin content in grains

The anthocyanin content of the rice grains of the selected F_4 lines was estimated using the differential pH (pH 1.0 and 4.5) method described by Fuleki and Francis (1968). Briefly, a few grains of each sample were de-husked and ground into a fine powder using mortars and pestles. 100 mg of the powder was weighed and transferred to individual 2 mL tubes for each sample. 1.5 mL of anthocyanin extractant {85:15 of Methanol: HCl (95% methanol and 1.5 N HCl)} was added to make the volume to 2 mL to each tube. The tubes were kept in an orbital shaker overnight at 250 RPM followed by centrifugation at 10,000 RPM for 10 min. The purple-coloured supernatants were collected in fresh 15 mL Falcon tubes. To the pellet, the anthocyanin extractant was added again and the previous supernatant extraction steps were repeated until it turned colourless. 1 mL extract was dispensed in a tube and the volume was made up to 15 mL with pH 1.0 buffer (25:67 v/v of 0.2 M KCl:1.5 N HCl). The same was done with the pH 4.5 buffer (100:60:90 of 1 M Sodium acetate:1 N HCl: H₂O). Using the *Spectroquant*[®] *Prove 300* spectrophotometer (Merck, Darmstadt, Germany), the absorbance of the pigment of these two solutions at $\lambda = 510$ nm was read and recorded. The same was done for all other samples. Modifying the formula given by Ranganna (1986), the total anthocyanin content was calculated as follows:

Total Anthocyanin (mg 100 g⁻¹) = {Total Absorbance at $\lambda_{510\text{ nm}}$ (pH 1.0) – Total Absorbance at $\lambda_{510\text{ nm}}$ (pH 4.5)} / 77.5 (absorbance of the solution containing 1 mg mL⁻¹ anthocyanin at pH 4.5) where,

Total Absorbance at $\lambda_{510\text{ nm}}$ = (Absorbance at $\lambda_{510\text{ nm}}$ x Volume made up for reading x Total volume of supernatant collected x 100) / (Volume of extract x Weight of sample in grams)

RESULTS AND DISCUSSION

Screening of Chakhao landraces for *Pi54* gene

The prevalence of many blast-resistant (R) genes has already been reported in many rice germplasm of India. *Pi54* is a blast-resistant gene that was first identified in an Indian rice cultivar called HR-22 (Kiwosawa and Murty 1969). It

has also been reported to impart total resistance against blast caused by *Magnaporthe oryzae* and its many strains in some instances (Sharma et al. 2005, Azizi et al. 2016). Additionally, it was found that by upregulating many transcription factors that mediate defence response genes such as WRKY6, WRKY45, NAC6, etc., *Pi54* was also able to induce moderate resistance against other diseases such as sheath blight (Singh et al. 2020). The positive allele for the *Pik-h* or *Pi54* gene with an amplicon size of 216 bp was detected in 118 landraces in India (Yadav et al. 2019). *Pi54MAS* is an allele-specific co-dominant marker developed so that it could target this particular InDel and produce 216 bp resistant or 356 bp susceptible amplicons (Ramkumar et al. 2011). In this study, out of the 40 Chakhao landraces that were screened for the presence of *Pi54* using *Pi54MAS* marker, 10 Chakhao genotypes were found to be positive (Table 1). Owing to its

Table 1. List of Chakhao landraces used for screening of *Pi54* in the present investigation. The presence of functional allele of *Pi54* was determined by the presence of 140bp with RM 206 primer and 216bp with *Pi54MAS* primers

Sl. No.	Sample	<i>Pi54MAS</i>	<i>Pi54</i>
1	ChK 1	-	-
2	ChK 2	-	-
3	ChK 3	-	-
4	ChK 4	-	-
5	ChK 5	216 bp (resistant)	Present
6	ChK 6	359 bp (susceptible)	Absent
7	ChK 7	216 bp (resistant)	Present
8	ChK 8	216 bp (resistant)	Present
9	ChK 9	-	-
10	ChK 10	216 bp (resistant)	Present
11	ChK 11	359 bp (susceptible)	Absent
12	ChK 12	-	-
13	ChK 13	216 bp (resistant)	Present
14	ChK 14	216 bp (resistant)	Present
15	ChK 15	-	-
16	ChK 16	359 bp (susceptible)	Absent
17	ChK 17	-	-
18	ChK 18	-	-
19	ChK 19	359 bp (susceptible)/ 216 bp (resistant)	Absent
20	ChK 20	359 bp (susceptible)	Absent
21	ChK 21	-	-
22	ChK 22	359 bp (susceptible)	Absent
23	ChK 23	-	-
24	ChK 24	359 bp (susceptible)	Absent
25	ChK 25	-	-
26	ChK 26	-	-
27	ChK 27	-	-
28	ChK 28	-	-
29	ChK 29	359 bp (susceptible)/ 216 bp (resistant)	Absent
30	ChK 30	-	-
31	ChK 31	-	-
32	ChK 32	-	-
33	ChK 33	216 bp (resistant)	Present
34	ChK 34	-	-
35	ChK 35	216 bp (resistant)	Present
36	ChK 36	-	-
37	ChK 37	359 bp (susceptible)	Absent
38	ChK 38	216 bp (resistant)	Present
39	ChK 39	216 bp (resistant)	Present
40	ChK 40	359 bp (susceptible)	Absent

superior grain quality, ChK13 with the positive allele of the *Pi54* gene was selected as the recipient parent in the present investigation.

Introgression of *OsSPL14* gene into Chakhao

Introgression is the process by which gene flow is allowed between different populations by transferring the desired gene into the other, which results in its improvement. Recently, the improvement of Chakhao Amubi by the introgression of a drought-tolerant QTL from cultivar Apo was reported (Kambale et al. 2022). Successful introgression of the *OsSPL14* gene in elite *indica* cultivars increasing in the grain number of the panicles of the crops has also been reported (Kim et al. 2018). We hybridized ChK13 and CR Dhan 307 to develop F_1 s and selfed them to ultimately develop a total of 147 F_4 lines. We used Pi54MAS as the marker for the screening of the *Pi54* gene in the F_4 lines, which produced an amplicon of size 216 bp (Figure 1a). For the *OsSPL14* gene, SPL14-12SNP marker was used with its positive allele giving an amplicon size of 302 bp (Figure 1b). Out of 147 F_4 lines, 32 lines were found positive for both genes, *Pi54* and *OsSPL14*, of which 16 lines (hereafter referred to as ChM series) were aromatic and black grain coloured while the remaining 16 lines were white grain coloured.

Lodging is one of the most common problems faced by farmers in the cultivation of Chakhao due to the high plant height nature. The average plant height of the 16 F_4 lines was 119.3 ± 1.132 cm with the shortest measuring 41.5 ± 1.598 cm and the tallest, 165 ± 2.764 cm, while the remaining 7 were of dwarf statures measuring well below 100 cm plant height. Due to their physical statures, these lines have therefore alleviated their phenotypic trait in addition to their already enhanced genetic characteristics. In a study conducted by Borah et al. (2018), the average grain length of Chakhao was found to be between 9.45 mm and 9.75 mm, grain width between 3.65 mm and 2.63 mm, and plant height ranged between 165.5 cm and 136.4 cm. In this study, we found that the average grain length was 8.48 mm, ranging from 7.32 mm to 9.17 mm, while the grain width ranged from 2.24 mm to 3.16 mm with an average of 2.73 mm. The grain thickness ranged from 1.19 mm to 2.02 mm with an average of 1.73 mm and 1000-grain weighed an average of 18.65 g, ranging from 10.62 g to 25 g. The average plant height was 119.3 cm with the shortest measuring 41.5 cm and the tallest, 165 cm. From this data, we could conclude that the general phenotypic characteristics of the selected lines are concurrent with those found in the Chakhao landraces in Manipur.

Background selection for advanced selected genotypes

We used a total of 293 SSR markers from the overall 12 rice chromosomes to check polymorphism between CHK 13

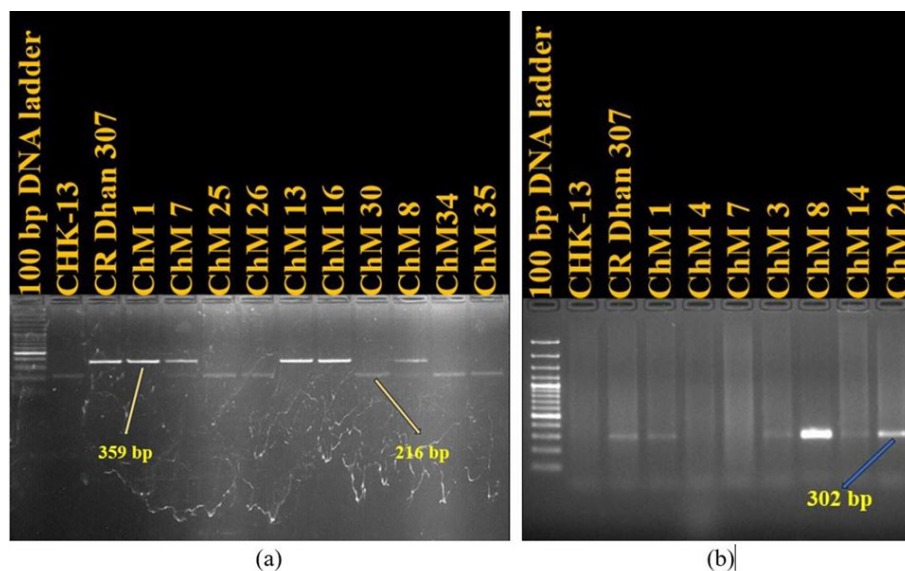


Figure 1. Foreground selection of F_4 lines using functional marker, Pi54MAS with amplicon size 216 bp (a) and *OsSPL14*-12SNP with 302 bp (b) corresponding to *Pi54* and *OsSPL14*, respectively.

and CR Dhan 307. Out of 293, 78 (26.62%) markers were found polymorphic between the two parental lines with the highest number of polymorphic markers found in chromosome 8 (12), followed by chromosome 1 (11), chromosome 4 (10), chromosome 5 (9), chromosome 7 (8), chromosome 2 (7), chromosome 3 (6), chromosome 6 (6), chromosome 11 (4), chromosome 9 (3) and the least in chromosome 10 (1) and chromosome 12 (1). The details of the 78 polymorphic data along with few gel images have been provided in supplementary file 1 and 2. Out of these 78 SSR markers, only 50 SSR markers (64.10%) were found informative across the 16 F₄ lines selected for background selection/genotyping (Figure 2). The polymorphism across these lines shows that the genomic contribution of the Chakhao parent is more than that of CR Dhan 307. All the 16 F₄ lines have more than 50% genomic similarity to ChK13, with ChM 142 having the highest with 81.6% of ChK13 and the lowest being both ChM 125 and ChM 63 with 64.3% of ChK13. These results revealed that the selected 16 lines were more phenotypically and genotypically similar to the Chakhao genotypes, which is desirable for the development of varieties as black rice.

Total anthocyanin content estimation

Chakhao has been reported to contain high contents of protein, fibre and minerals. Furthermore, higher content of polyphenols and phytates has been reported in Chakhao as compared to white rice. The rich anthocyanin content of Chakhao contributes to its many nutraceutical benefits, making it attractive in the market. The anthocyanin that is accumulated on the pericarp is responsible for the blackish grain colour of black rice varieties (Rahman et al. 2013). Asem et al. (2015) found that the total anthocyanin content of two popular Chakhao varieties in Manipur, Chakhao

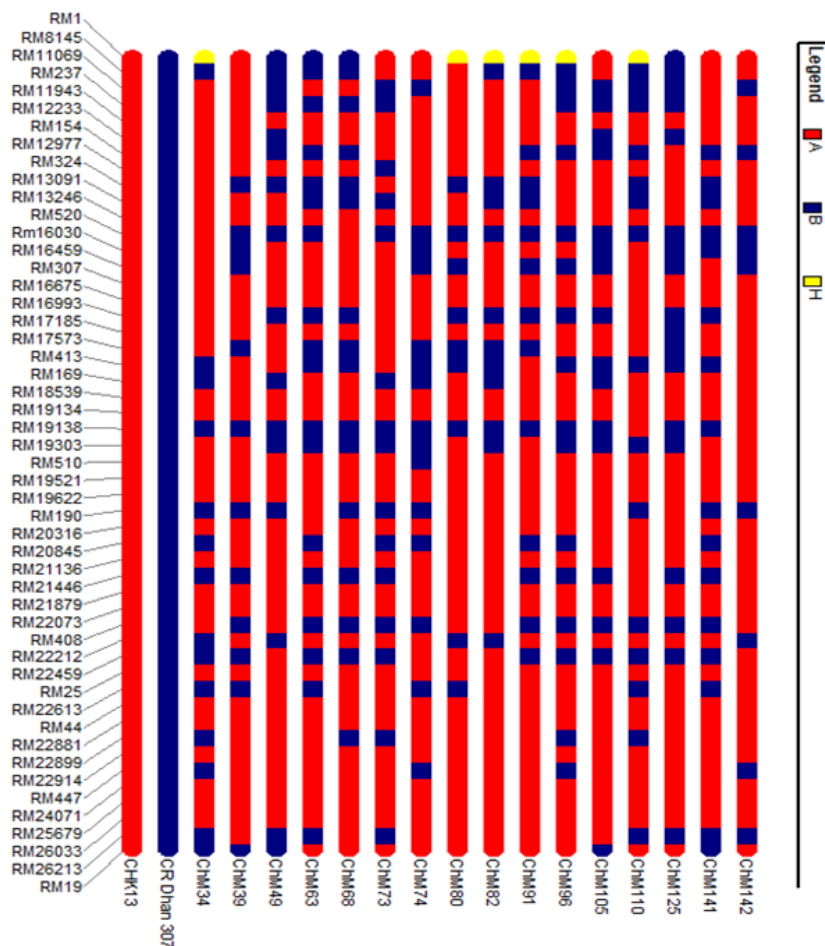


Figure 2. Graphical genotype of 16 F₄ lines showing parental genome contribution using 50 polymorphic SSR markers. The ChK 13 alleles are denoted by the letter A and the colour red, the CR Dhan 307 alleles are denoted by the letter B and the blue colour, while the heterozygote alleles are denoted by the letter H and yellow colour.

Poireiton and Chakhao Amubi, were 740 mg kg⁻¹ and 692 mg kg⁻¹, respectively. The total anthocyanin content among the selected 16 F₄ genotypes ranged from 30.19±2.19 to 240.31±2.62 mg 100g⁻¹ dried weight. Similarly, the total anthocyanin content among pigmented and aromatic germplasm of Manipur was reported with a range from 29.8 to 275.8 mg 100g⁻¹ DW (Bhuvanewari et al. 2020). In the present investigation, ChM 68 showed the highest anthocyanin content, 240.31±2.62 mg 100 g⁻¹ sample, which is 118.96 mg 100g⁻¹ more as compared to the recipient parent, CHK13 (121.35±3.32 mg 100 g⁻¹), whereas ChM 73 showed the least, with 30.19 ± 2.19 mg 100 g⁻¹. Compared to the recipient parent, CHK13, the other selected F₄ with higher anthocyanin content are ChM 105 (166.45±2.74 mg 100 g⁻¹), ChM 91 (124.65±3.28 mg 100 g⁻¹), and ChM 49 (122.19±4.65 mg 100 g⁻¹) (Table 2). Intriguingly, ChM 68 with the highest anthocyanin content among the 16 F₄ lines was a semi-dwarf plant that would be suitable to be released as an improved cultivar of black rice in Manipur. Furthermore, the black colour of the grains and the high anthocyanin content in all 16 selected lines indicated that we could retain more of the Chakhao genome than the other parent. This statement can also be supported by background selection, which showed that the genomic contribution of Chakhao varied between 64.3 and 81.6% in the selected lines, which is more than half of the total genome of the Chakhao.

Table 2. Anthocyanin content of the selected 16 F₄ lines along with recipient parent. For each line, duplicate samples were used for the estimation

Serial No.	Sample	Total Anthocyanin content (mg 100 g ⁻¹ sample)
1	ChM 34	27.68 ± 0.27
2	ChM 39	61.16 ± 2.74
3	ChM 49	122.19 ± 4.65
4	ChM 63	60.97 ± 0.27
5	ChM 68	240.31 ± 2.62
6	ChM 73	30.19 ± 2.19
7	ChM 74	110.52 ± 5.75
8	ChM 80	64.06 ± 0.27
9	ChM 82	108.97 ± 0.96
10	ChM 91	124.65 ± 3.28
11	ChM 96	50.35 ± 0.27
12	ChM 105	166.45 ± 2.74
13	ChM 110	45.29 ± 2.74
14	ChM 125	30.87 ± 4.38
15	ChM 141	119.81 ± 3.56
16	ChM 142	108.77 ± 4.38
17	CHK13	121.35±3.32

CONCLUSION

The beneficial properties of Chakhao are more than a handful, which has made its demand rise exponentially in the markets. To be able to meet this, the genetic improvement of Chakhao which will ultimately increase its yield can be one of the long-term solutions. In this current study, we were able to successfully develop improved Chakhao lines with both the genes of the blast R-gene, *Pi54*, and the high-yielding gene, *OsSPL14*. In addition to the introgression of these two important genes, the selected lines have also shown high anthocyanin contents. Apart from these improvements, some of these lines were phenotypically short, which will be even more advantageous than their tall counterparts as they might be able to withstand the age-old problem Chakhao has faced with lodging. If successfully bred, these lines can prove to be a long-awaited solution to the low production dilemma farmers face in the cultivation of Chakhao.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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