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Using Molecular Markers in Breeding Rice with Colored Pericarp for Blast Resistance

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ABSTRACT

The study aimed to develop rice (Oryza sativa L.) varieties with colored pericarp and blast (Magnaporthe oryzae) resistance using molecular marker-assisted selection. The study used late-hybrid lines and varietal samples selected for the presence of rice blast resistance genes (Pi): *Pi-1, Pi-9, Pi-40*, and *Pi-54*. Gene identification was carried out by polymerase chain reaction (PCR) using simple sequence repeat (SSR) markers. Of the 38 genotypes analyzed, 14 hybrid lines and 7 varieties each contained 3 resistance genes, providing long-term resistance to the pathogen. A variety of red-grain glutinous rice, Almavita, containing the Pi-40 gene and having a wide spectrum of resistance, was developed and recommended for State Variety Testing. Agronomic evaluation demonstrated its high yield, resistance to lodging, and low grain loss. In conclusion, molecular markers were effectively used to accelerate the selection of resistant genotypes. The *Almavita* variety shows great value for practical breeding and can help to reduce dependence on imported red rice. The conducted comprehensive research identified promising late-generation hybrid lines of rice with colored pericarp and provided the necessary data to submit the red-grain glutinous rice variety *Almavita*, obtained by individual selection method from the F5 Vita/Fatima hybrid in the Balkhash District, Almaty Region, Kazakhstan, to State Variety Testing.

Keywords: Rice with colored pericarp, Blast disease, Marker-assisted selection (MAS), Resistance genes, Almavita variety.

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INTRODUCTION

Rice (*Oryza sativa* L.) is a key food crop, but its productivity is severely hampered by phytopathogenic diseases (Amoo et al., 2023; Riaz et al., 2025). The most notable of these diseases is blast, which is caused by the fungus *Magnaporthe oryzae* and can cause losses of 10–20% in typical years and up to 100% under epiphytotic conditions in the major rice-growing regions (Dean et al., 2005; Poonsin & Parinthawong, 2020; Martín-Cardoso et al., 2024). The most effective method of controlling this rice disease is the development of resistant varieties (Ellur et al., 2025; Song et al., 2025). However, varietal resistance is often lost after a few years due to environmental changes and the emergence of new more aggressive strains of *Pyricularia oryzae* (Huang et al., 2011; Poonsin & Parinthawong, 2020; Kuanbay et al., 2025). Gene introduction and pyramiding are efficient ways to create blast-resistant rice varieties (Amirova et al., 2024; Mynbayeva et al., 2024). Molecular markers make it possible to detect resistance genes in rice genotypes and to carry out gene introduction and pyramiding, which accelerates the process of creating rice varieties resistant to *M. oryzae* (Kazkeev et al., 2019). Due to the high variability of rice blast, new rice varieties with long-term resistance (R) need to be developed using introgression and molecular markers. At present, there are over 100 known blast resistance genes in rice (Huang et al., 2011; Xiao et al., 2019; Liu et al., 2021).

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Most of the world's rice acreage is occupied by white-grain varieties (Tong et al., 2019). However, the wild ancestors of cultivated rice had a colored pericarp (Guo et al., 2025; Zhao et al., 2025). Today, along with white-grain rice, the leading rice-growing countries are also creating and cultivating varieties with colored grain pericarp. Red rice is rich in nutrients and antioxidants, such as phenolic acids, flavonoids, γ-orezanol, anthocyanins, proanthocyanidins, etc (Mustikarini et al., 2020). The antioxidants in red rice help to reduce free radicals. Thus, rice with colored pericarp is used not only in cooking, but also in medicine as a biologically active supplement in the treatment of diabetes, atherosclerosis, allergies, and even cancer (Jain et al., 2019; Manzoor et al., 2023; Tan et al., 2023). In countries that cultivate rice traditionally, red and black rice are grown alongside white rice varieties for use as a dietary and therapeutic product (Zhang et al., 2022). These types of rice are consumed without grinding, preserving the nutrients and biologically active substances valued by these cultures. Rice with colored pericarp also has a higher antioxidant activity than white rice (Zelenskaya et al., 2018). According to the recent studies, we understand that pericarp colors (PC) are complex genetic traits, and the mechanisms surrounding them are yet to be understood. Yang et al. (2022) investigated the notion that light is a key factor influencing the expression of flavonoid biosynthetic genes, implying that differences in light and temperature may also cause variations in the degree of pericarp color (DPC) of rice planted in two locations. Their findings revealed that the white or colored pericarp of 442 rice accessions remained constant, whereas the DPC of colored rice accessions varied across environments, implying that the presence and absence of PC in rice is a qualitative trait unaffected by the environment, whereas the DPC of colored rice is a quantitative trait influenced by the environment. Apart from its antioxidant and blast-resistant traits, rice with pericarp colors has been shown to possess nutritional and yield-related traits. Kiran et al. (2024) concluded in their study that colored pericarp provides health benefits against lifestyle diseases. Sh et al. (2021) found that colored rice cultivars contain polyphenols that promote health by lowering the concentration of free radicals that damage cells. Anthocyanin is the flavonoid responsible for the red, purple, blue, and black colors of rice types. These chemicals exhibit health benefits such as antioxidant, anti-cancerous, anti-inflammatory, and hypoglycemic activities (Kemashalini et al., 2018). Yamsaray et al. (2022), in their study focused on the yield and nutritional properties of improved red pericarp Thai rice varieties, concluded that *PTT1*, a brown rice bran, produced the highest yield, and followed by *R684-13-15*. The black rice cultivars contained the most protein and lipids, followed by the red and brown rice variants (Yi et al., 2020). Furthermore, red rice cultivars exhibited the highest antioxidant activity, followed by black and brown rice kinds. *R2535-8-10*, a mutant red rice line, had the greatest values of TPC and FRAP, as well as significant DPPH radical scavenging activity; *R684-13-15* had high yield, TPC, FRAP, and DPPH levels. Both red rice types (*R2535-8-10* and *R68413-15*) should be suggested for cultivation because of their great nutritional content. Ecologically, rice with colored pericarps, like red or black rice, has ecological relevance due to its higher antioxidant content, such as anthocyanins and proanthocyanidins, which are highly beneficial to human health and may offer protection against environmental stressors (Yang et al., 2022).

Rice genetic resources are widely collected and preserved, forming the basis for developing improved cultivars. Colored pericarp varieties such as red, black, and purple rice are particularly valuable, originating from both cultivated forms and wild or weedy relatives that provide traits like resistance to shattering, stress tolerance, and enhanced nutritional qualities (Nair, 2019; Mukhametov et al., 2024). Their use in breeding programs both conserves genetic diversity and supports the creation of resilient, high-quality varieties. Modern breeding combines artificial hybridization, which allows controlled transfer of desirable traits, with the selection of spontaneous hybrids among white, red, and black rice. These natural crosses generate novel genetic variability, offering opportunities for stress-resistant and high-yield cultivars. Integrating deliberate crossing with natural gene flow emphasizes the importance of broad genetic resources and the balance of traditional and advanced methods in rice improvement (Zelenskaya et al., 2018).

Asian rice (*Oryza sativa* L.) is a primary staple food, central to the diet of billions worldwide, particularly in Asia. Although rich in carbohydrates, its husk is largely indigestible due to fibrous components such as cellulose and lignin. For consumption, brown rice is polished into white rice, removing the pericarp, seed coat, nucellus, and aleurone, which together form rice bran. Oil extracted from this bran (RBO) is valued for its bioactive compounds, including γ-oryzanol, vitamin E, and phytosterols, giving it antioxidant, cholesterol-lowering, and cardioprotective properties (Karimov, 2009; Bhat et al., 2020; Vassilina et al., 2024).

Pigmented rice varieties are increasingly recognized as functional foods. Their coloration reflects high levels of anthocyanins and proanthocyanidins, which provide antioxidant, anti-inflammatory, and antidiabetic effects (Peng et al., 2020). Wild progenitors of cultivated rice (*Oryza nivara* and *Oryza rufipogon*) naturally bore colored pericarps, while white rice emerged through domestication and selection for processing ease. Today, pigmented rice is gaining importance as both a nutritional resource and a bridge between traditional diets and modern functional food innovation. The color of rice grain (associated with antioxidant activity) is the result of the pigmentation of the sclerenchyma and (or) pericarp greatly, including purple (violet-black), red, brown, yellow, and incomplete spotty pigmentation (He et al., 2021; Chen et al., 2022). Rice grains rich in anthocyanins are also recognized as an excellent source of natural and safe food coloring (Tumanian et al., 2020; Papillo et al., 2018; Xiang et al., 2022). To date, there has been no dedicated breeding studies in Kazakhstan aimed at producing varieties of rice with colored pericarp. As a result, Kazakhstan does not currently have domestic forms of red and black rice. Consequently, the need for this type of rice is met by imports. However, imported samples of black rice, for example, cost 5–6 times more than traditional white rice, which significantly limits its availability to the population. Therefore, developing Kazakh varieties of rice with colored pericarp by means of a combination of classical and molecular-genetic breeding methods appears to be an urgent and socially significant task. The purpose of this study was to identify and select promising varieties and hybrid lines of rice with colored pericarp with genetic resistance to *M. oryzae* by means of marker-assisted selection (MAS). Rationale for gene selection and local relevance. We prioritized *Pi-1, Pi-9, Pi-40*, and *Pi-54* because they are widely reported as broad-spectrum blast R-genes and are supported by validated markers (e.g., *Pi-40* linked to *9871.T7E2b/MSM6*; *Pi-9* cloned at the *Pi2/Pi9* locus) that facilitate MAS. International bioassays show Pi-40 confers durable, broad resistance across diverse isolates, while *Pi-9* and *Pi-54* repeatedly broaden resistance spectra when pyramided; *Pi-1* complements these by expanding race coverage. Importantly for Kazakhstan, recent IPBB programs and regional screens have selected and introrse these same genes (including *Pi-1, Pi-40, Pi-54*) into local backgrounds, reflecting their effectiveness against prevalent Kazakh M. oryzae populations and practical utility in domestic breeding.

MATERIALS & METHODS

Field work was conducted in the Balkhash District, Almaty Region, Kazakhstan, and laboratory tests were conducted at the Institute of Plant Biology and Biotechnology. The research involved a comprehensive set of rice accessions representing both pigmented and non-pigmented forms, as well as advanced hybrid lines and dihaploid (DH) materials derived from them. The experimental material included three main categories: (a) parental accessions, (b) red-grain hybrids, and (c) anthocyanin-pigmented hybrids:

a) initial parental forms of black and red rice (black rice – Mavr, Black Rice; red rice – Yir 5815.

b) ***Red hybrids***. Several F_7 hybrid generations were obtained using Yir 5815 crossed with different cultivated varieties: F_7 Yir 5815/Pak Lee *(var.sundensis Koern);* F_7 Yir 5815/Pak Lee *(var.subpyrocarpa Gust);* F_7 Yir 5815/Bakanassky *(var.sundensis Koern);* F_7 Yir 5815/Bakanassky *(var.pyrocarpa Alef);* F_7 Yir 5815/Marjan *(var.pyrocarpa Alef).* DH2 F_2 Yir 5815/Marjan *(var. pyrocarpa Alef)*; Almavita. In addition, a DH_2 F_2 hybrid (*Yir 5815/Marjan*, var. *pyrocarpa* Alef) and the cultivar *Almavita* were also included in this group. These hybrids served as valuable material for assessing the inheritance of red pericarp and associated agronomic performance.

c) ***Anthocyanin-colored rice hybrids.*** A larger set of hybrid lines derived from black rice (Mavr, Black Rice) was studied to evaluate the inheritance of anthocyanin pigmentation. These included: F_8 Black Rice/Yantar anthoc.pigm.; F_8 Black Rice/Yantar *(var.pseudovialonica Vasc);* F_8 Black Rice/Yantar *(var.nigrispina Port);* F_6 Black Rice/ Sprint *(var. pseudovialonica Vasc);* F_6 Black Rice/ Sprint *(var. pyrocarpa Alef);*F_8 Black Rice/Bakanassky *(var. pseudovialonica Vasc);* F_8 Black Rice/Bakanassky *(var.Desvauxii Koern);* F_8 Black Rice/Bakanassky *(var.Eediana Koern);* F_8 Black Rice/Bakanassky *(var.para-Gastrol Port);* F_8 Black Rice/Marjan; F_8 Black Rice/Marjan *(var. pyrocarpa Alef);* F_8 Black Rice/Marjan *(var.subpyrocarpa Gust);* F_8 Mavr/Kurchanka *(var.pyrocarpa Alef);* F_8 Mavr/Kurchanka *(var.sundensis Koern);* F_8 Mavr/Pak Lee *(var.bansmatica Koern);* F_8 Mavr/Bakanassky *(var. Desvauxii Koern);* DH2 F_2 Black Rice/Bakanassky. This diverse collection of hybrids made it possible to examine the expression of pigmentation across multiple genetic backgrounds, track inheritance patterns, and evaluate agronomic traits such as productivity, adaptability, and resistance to environmental stress.

d) white-grain rice varieties: Yantar, Marjan, Bakanassky, Pak Lee, Kurchanka, Sprint, and late hybrid lines obtained with their participation.

Phenological observations of the hybrids were performed according to the method of Erygin & Krasnook (1965).

Total amylose content in rice grain was determined usinf the method described by Juliano (1971).

Biochemical Analysis of Spare Proteins from Rice

Spare proteins were extracted with Tris-HCl and phosphate buffer pH 6.8 containing SDS Na, mercaptoethanol, glycerol, and bromphenol blue dye. Fractionation was performed on 10% polyacrylamide gel following a modified Laemmli method (Bulatova, 1985).

The experiments were repeated three times, and the final result was presented as Mean ± Standard Deviation, which were calculated in SPSS v 29. Lodging was noted at maturity, and grain loss was calculated as the percentage of undeveloped caryopses per panicle.

Blast Resistance Assessment (Genotypic)

Blast resistance was inferred from PCR/SSR genotyping at Pi-1, Pi-9, Pi-40, and Pi-54 using the specified locus-linked markers, with presence/absence calls based on expected amplicon sizes relative to positive and negative controls.

The use of DNA Markers in Breeding Rice with Colored Pericarp

Blast resistance genes were identified in the samples using markers for the *Pi-1, Pi-9, Pi-40, Pi-54,* and *Pita* genes and optimized PCR parameters. Genomic DNA isolation from rice plants was performed by the CTAB method (Murray & Thompson, 1980). The DNA was isolated from chlorophyll-free seven-day-old seedlings obtained by incubation on moistened filter paper in the dark at 25–27°C. Fresh leaf samples were placed in microcentrifuge tubes and rubbed with 600μL CTAB extraction buffer (*1M Tris pH8.0; 1M NaCl; 0.5M EDTA pH 8.1; 2% STAB*) heated to 65°C. The samples were incubated at 65°C for one hour, after which an equal volume of chloroform was added and incubated while stirring for 20minutes. The samples were centrifuged for 20min at 5,000rpm, 300-400µL of the supernatant were transferred to a clean microtube, 500µL of isopropyl alcohol were added to precipitate the DNA, and the samples were incubated for 30min at –20°C. After incubation, centrifugation was performed again for 10min at 5,000rpm. The supernatant was carefully removed, the DNA precipitate was washed with 500µL of 70% ethanol, dried for 60min at room temperature, and dissolved in 100µL of 1xTE buffer. Next, 1µL of RNase (Sigma) (10mg/ml) was added and incubated at 37°C for 30min. Following this, 1/10 of the volume (10µL) of 5M sodium acetate was added to the sample and left overnight at –20°C. The next day, the precipitate was centrifuged at 5,000rpm for 15min, the precipitate was washed twice with 70% ethanol, and the white precipitate was dissolved in 100µL TE buffer. The concentration of the isolated DNA was determined by spectrophotometry and by the intensity of DNA staining with ethidium bromide in agarose gel. The total volume of the reaction mix was 15µL, including 1.5µL 10xPCR buffer (10mM Tris-HCl, pH = 7.4; 50mM; 1.5mM $MgCl_2$), 1unit of Taq polymerase; 10mM of dNTP (Sigma); 10mM of each primer, and 100–200ng of the analyzed DNA. In this study, we used Thermus aquaticus (Taq) thermostable DNA polymerase (BioLabs, New England). To determine its quality and concentration, the isolated DNA was examined on a spectrophotometer and separated in a 1.0% agarose gel with ethidium bromide.

Blast resistance genes were identified in the studied varieties and late generation hybrid lines by means of PCR using the following molecular markers: RM 224 and RM 1233 (*Pi-1*), MSM6 and 9871.T7E2b (*Pi-40*), 195R-1 and NMSMPi-9 (*Pi-9*), and TRS26 and Pikh MAS (*Pi-54*). Their nucleotide sequences and annealing temperatures are provided in Table 1 (Gouda et al., 2012; Shilov et al., 2018). PCR was performed on a T100 thermal cycler (BioRad, USA). PCR products were separated on 2% agarose gel.

PCR Amplification Parameters

Reactions (15µL) were run with the following thermal profile: initial denaturation 95°C for 3min; 35 cycles of 95°C for 30s, T_a for 30s (primer-specific annealing temperatures as in Table 1), and 72°C for 45s; final extension 72°C for 5min. Final reagent concentrations were: 1× buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2mM each dNTP, 0.2µM each primer, 1 U Taq polymerase, and 100–200ng template DNA. Stock solutions of primers and dNTPs were 10 mM, but final working concentrations were as stated here.

Gel Electrophoresis and Visualization

Amplicons were resolved on 2% agarose in 1× TBE, run at 90 V for 45–60min alongside a 100 bp DNA ladder; gels were stained with ethidium bromide and imaged on a GelDoc XR+ (Bio-Rad). Each run included positive controls (*Pi-1: IR BL1-CL; Pi-40: KPD-7712; Pi-54: IR 04636*) and a negative control (Nipponbare), matching the figure panels, to validate expected band sizes and specificity.

RESULTS AND DISCUSSION

Using Molecular Markers in Breeding Rice with Colored Pericarp

One of the most effective methods to protect plants against diseases is breeding resistant varieties that carry multiple resistance genes. MAS can be used to test rice breeding lines with colored pericarp for several blast resistance genes. For PCR analysis, we used molecular markers closely related to the corresponding genes: RM 224 and RM 1233 (*Pi-1*), MSM6 and 9871.T7E2b (*Pi-40*), 195R-1 and NMSMPi-9 (*Pi-9*), and TRS26 and Pikh MAS (*Pi-54*). The amplification of PCR products of rice hybrids with colored pericarp and initial forms is in Fig. 1–4. The *Pi-1* blast resistance gene was identified by the presence of the RM 224 and RM 1233 markers together. All tested samples had an amplicon of 137bp corresponding to the RM 224 marker, while the 170bp amplicon was not detected using the RM 1233 marker (Fig. 1). All genotypes under study were heterozygous for this locus, since the recessive gene corresponding to the amplicon size of 400bp was detected in the variety *Nippon bare* (does not contain R genes), which was used as a negative control. Genotypes that are homozygous for a certain gene are known to have higher resistance compared to heterozygous genotypes, so it is advisable to target only homozygous lines for breeding to create resistant varieties.

Table 1: Analyzed molecular markers for *Pi* genes

Molecular marker	Gene	Nucleotide sequence	PCR product size (bp)	Annealing, T (°C)	Localization on the chromosome
RM224	*Pi-1*	F — ATCGATCGATCTTCACGAGG R — TGCTATAAAAGGCATTCGGG	137	56	11
RM1233		F — AATAGGCCTGGAGAGAATTTCC R — CCTTATAAGCCGTCTCGATCC	170	56	11
MSM6	*Pi-40*	F — TGCTGAGATAGCCGAGAAATC R — GCACCCTTTTCGCTAGAGG	256	58	6
9871.T7E2b		F — CAACAAACGGGTCGACAAAGG R — CCCCCAGGTCGTGATACCTTC	641	58	6
195R-1	*Pi9*	F — ATGGTCCTTTATCTTTATTG R — TTGCTCCATCTCCTCTGTT	2000	55	6
NMSMPi9-1		F — CGAGAAGGACATCTGGTACG R — GAGATGCTTGGATTTAGAAGAC	168	55	6
TRS26	*Pi-54*	F — GGAGAGCCAATCTGATAAGCA R — CAACAAGAGAGGCAAATTCTCA	266	54	11
Pikh MAS		F — CAATCTCCAAAGTTTTCAGG R — GCTTCAATCACTGCTAGACC	216	54	11

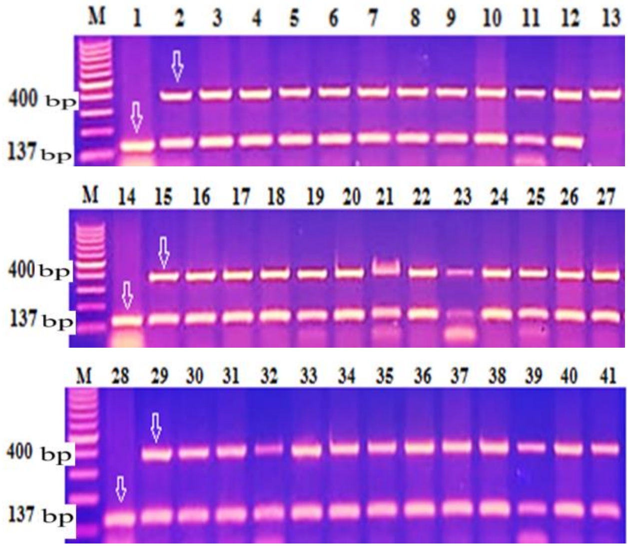

Fig. 1: Amplification of PCR products of rice hybrids with colored pericarp with the initial forms for the presence of the *Pi-1* gene. Plate 1. M — marker, 1 — standard (IR BL1-CL) , 2 — F_7 Yir 5815/ Bakanassky *var.sundensis Koern,* 3 — F_7 Yir 5815/ Bakanassky *var.pyrocarpa Alef*, 4 — F_7 Yir 5815/Pak Lee *var.sundensis Koern*, 5 — F_7 Yir 5815/Pak Lee *var.subpyrocarpa Gust*, 6 — F_7 Yir 5815/Pak Lee *var.pyrocarpa Alef*, 7 — F_7 Yir 5815/Marjan *var.pyrocarpa Alef*, 8 — DH2 F_2 Yir 5815/Marjan *var.pyrocarpa Alef*, 9 — Yir 5815, 10 — Bakanas, 11 — Pak Lee, 12 — Marjan, 13 — negative control (Nippon bare); Plate 2. M — marker, 14 — standard, 15 — F_8Black Rice/Marjan, 16 — F_8 Black Rice/Marjan *var.pyrocarpa Alef*, 17 — F_8Black Rice/Marjan *var.subpyrocarpa Gust*, 18 — F_8 Black Rice/Bakanassky *var.pseudovialonica Vasc*, 19 — F_8 Black Rice/ Bakanassky *var.Desvauxii Koern*, 20 — F_8 Black Rice/ Bakanassky *var.Eediana Koern*, 21 — F_8 Black Rice/Bakanassky *var.para-Gastrol Port*, 22 — F_8 Black Rice/Viola *var.Desvauxii Koern*, 23 — DH2 F_2 Black Rice/Bakanassky, 24 — Black Rice, 25 — Marjan, 26 — Bakanassky, 27 — Viola; Plate 3. M — marker, 28 — standard, 29 — F_8 Black Rice/ Yantar anthoc.pigm., 30 — F_8 Black Rice/Yantar *var.nigrispina Port*, 31 — F_8 Mavr/ Kurchanka *var.pyrocarpa Alef*, 32 — F_8 Mavr/ Kurchanka *var.sundensis Koern*, 33 — F_8 Mavr/ Pak Lee *var.bansmatica Koern*, 34 — F_8 Mavr/Bakanassky *var.Desvauxii Koern*, 35 — Black Rice, 36 — Yantar, 37 — Mavr, 38 — Kurchanka, 39 — Pak Lee, 40 — Bakanassky, 41 — Almavita.

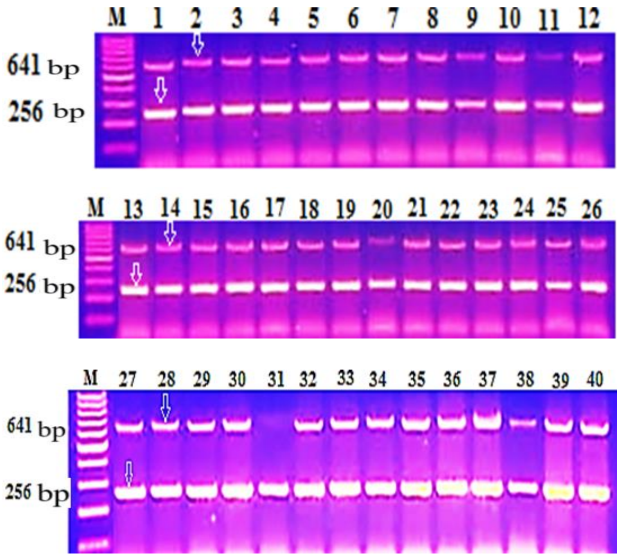

Fig. 2: Amplification of PCR products of rice hybrids with colored pericarp with the initial forms for the presence of the *Pi-40* gene. Plate 1. M — marker, 1 — standard (KPD 7712), 2 — F_7 Yir 5815/Bakanassky *var.sundensis Koern*, 3 — F_7 Yir 5815/ Bakanassky *var.pyrocarpa Alef*, 4 — F_7 Yir 5815/Pak Lee *var.sundensis Koern*, 5 — F_7 Yir 5815/Pak Lee *var.subpyrocarpa Gust*, 6 — F_7 Yir 5815/Pak Lee *var.pyrocarpa Alef*, 7 — F_7 Yir 5815/Marjan *var.pyrocarpa Alef*, 8 — DH2 F_2 Yir 5815/Marjan *var.pyrocarpa Alef*, 9 — Yir 5815, 10 — Bakanassky, 11 — Pak Lee, 12 — Marjan; Plate 2. M — marker, 13 — standard, 14 — F_8 Black Rice/Marjan, 15 — F_8 Black Rice/Marjan *var.pyrocarpa Alef*, 16 — F_8 Black Rice/Marjan *var.subpyrocarpa Gust*, 17 — F_8 Black Rice/Bakanassky *var.pseudovialonica Vasc*, 18 — F_8 Black Rice/ Bakanassky *var. Desvauxii Koern*, 19 — F_8 Black Rice/Bakanassky *var.Eediana Koern*, 20 — F_8 Black Rice/Bakanassky *var.para-Gastrol Port*, 21 — F_8 Black Rice/Viola *var.Desvauxii Koern*, 22 — DH2 F_2 Black Rice/Bakanassky, 23 — Black Rice, 24 — Marjan, 25 — Bakanassky, 26 — Viola; Plate 3. M — marker, 27 — standard, 28 — F_8 Black Rice/Yantar anthoc.pigm., 29 — F_8 Black Rice/Yantar *var.nigrispina Port*, 30 — F_8 Mavr/Kurchanka *var.pyrocarpa Alef*, 31 — F_8 Mavr/ Kurchanka *var.sundensis Koern*, 32 — F_8 Mavr/Pak Lee *var.bansmatica Koern*, 33 — F_8 Mavr/ Bakanassky *var.Desvauxii Koern*, 34 — Black Rice, 35 — Yantar, 36 — Mavr, 37 — Kurchanka, 38 — Pak Lee, 39 — Bakanassky, 40 — Almavita.

The *Pi-40* gene was detected based on SSR markers MSM6 and 9871.T7E2b. As a result, all samples were found to have the *Pi-40* blast resistance gene. Both desired amplicons with the sizes of 256bp and 641bp corresponding to the products of MSM6 and 9871.T7E2b markers were detected (Fig. 2). The only exception was one hybrid F_8 *Mavr/ Kurchanka var. sundensis Koern*, which showed only one of the sought amplicons of 256bp in size, which corresponds to the PCR product of the MSM6 marker. The 195R-1 and NMSMPi9-1 markers were used to identify the *Pi-9* gene. Hybrids *F7 Yir 5815/Pak Lee var.sundensis Koern* and *F8 Black Rice/Marjan var.subpyrocarpa Gust* and *variety Pak Lee* were heterozygous. *Almavita* was found to have a recessive allele of the gene with an amplicon size of 250bp like the negative control variety *Nippon bare*. The other lines and varieties were homozygous for the *Pi-9* gene with one amplicon of 168bp corresponding to the NMSMPi9-1 marker closely related to the *Pi-9* gene (Fig. 3).

PCR for the *Pi-54* gene using markers TRS26 and Pikh MAS shows that the studied microsatellite loci are represented by several allelic variants. Samples under numbers 2–5, 7–9, 32, 36, and 40 are heterogeneous with amplicons of 216bp and 400bp. The remaining lines 6, 10, 12, 13–31, 33–35, and 37–39 had 216bp and 266bp amplicons corresponding to Pikh MAS and TRS26 markers, the same as the positive control under number 1. Only sample 11 had one amplicon of 216bp. In addition, hybrids 32, 36, and 40 were found to have a 150bp amplicon (Fig. 4).

The screening of genotypes for the four *Pi* resistance genes shows that the initial varieties and hybrid lines of rice with colored pericarp have from 1 to 3 blast resistance genes (Table 2).

Blast-resistant rice samples were thus selected for further research using MAS. According to the strategy of blast resistance, long-term resistance to *M. oryzae* is ensured by the presence of 3–5 or more genes in the genotype (Suh et al., 2009). Of the rice genotypes studied, seven initial varieties and 14 hybrid lines with colored pericarp of late generation (Table 3) have three blast resistance genes each. The proposed variety Almavita has one *Pi-40* gene. However, research suggests that the *Pi-40* gene provides a wide spectrum of resistance, which proves its promise for breeding programs to develop varieties resistant to the population of *M. oryzae* (Suprun et al., 2016). In this study we evaluated Pi-1, Pi-9, Pi-40 and Pi-54 alleles, all of which have been cited as

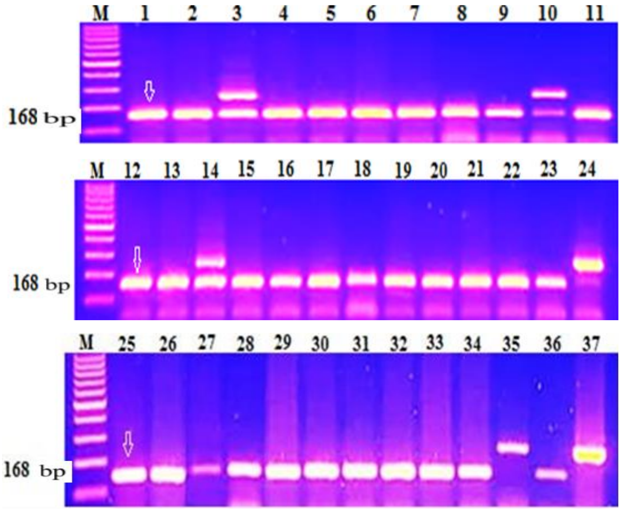

Fig. 3: Amplification of PCR products of rice hybrids with colored pericarp with the initial forms for the presence of the *Pi-9* gene. Plate 1. M — marker, 1– F_7 Yir 5815/Bakanassky *var.sundensis Koern*, 2 — F_7 Yir 5815/ Bakanassky *var.pyrocarpa Alef*, 3 — F_7 Yir 5815/Pak Lee *var.sundensis Koern*, 4 — F_7 Yir 5815/Pak Lee *var.subpyrocarpa Gust*, 5 — F_7 Yir 5815/Pak Lee *var.pyrocarpa Alef*, 6 — F_7 Yir 5815/Marjan *var.pyrocarpa Alef*, 7 — DH2 F_2 Yir 5815/Marjan *var.pyrocarpa Alef*, 8 — Yir 5815, 9 — Bakanas, 10 — Pak Lee, 11 — Marjan; Plate 2. M — marker, 12 — F_8Black Rice/Marjan, 13 — F_8 Black Rice/Marjan *var.pyrocarpa Alef*, 14 — F_8Black Rice/Marjan *var.subpyrocarpa Gust*, 15 — F_8 Black Rice/Bakanassky *var.pseudovialonica Vasc*, 16 — F_8 Black Rice/ Bakanassky *var.Desvauxii Koern*, 17 — F_8 Black Rice/ Bakanassky *var.Eediana Koern*, 18 — F_8 Black Rice/Bakanassky *var.para-Gastrol Port*, 19 — F_8 Black Rice/Viola *var.Desvauxii Koern*, 20 — DH2 F_2 Black Rice/Bakanassky, 21 — Black Rice, 22 — Marjan, 23 — Bakanassky, 24 — Viola; Plate 3. M — marker, 25 — F_8 Black Rice/Yantar anthoc.pigm., 26 — F_8 Black Rice/Yantar *var.nigrispina Port*, 27 — F_8 Mavr/ Kurchanka *var.pyrocarpa Alef*, 28 — F_8 Mavr/ Kurchanka *var.sundensis Koern*, 29 — F_8 Mavr/ Pak Lee *var.bansmatica Koern*, 30 — F_8 Mavr/ Bakanassky *var.Desvauxii Koern*, 31 — Black Rice, 32 — Yantar, 33 — Mavr, 34 — Kurchanka, 35 — negative control (Nippon bare), 36 — Bakanassky, 37 — Almavita.

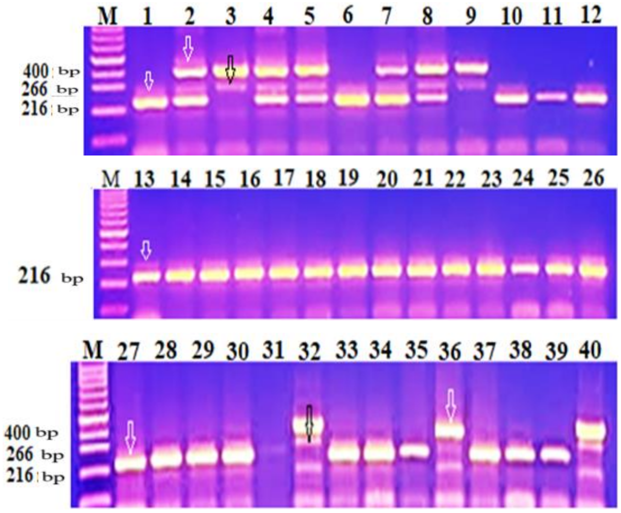

Fig. 4: Amplification of PCR products of rice hybrids with colored pericarp with the initial forms for the presence of the *Pi-54* gene. Plate 1. M — marker, 1 — standard (IR 04636), 2 — F_7 Yir 5815/Bakanassky *var.sundensis Koern*, 3 — F_7 Yir 5815/Bakanassky *var.pyrocarpa Alef*, 4 — F_7 Yir 5815/Pak Lee *var.sundensis Koern*, 5 — F_7 Yir 5815/Pak Lee *var.subpyrocarpa Gust*, 6 — F_7 Yir 5815/Pak Lee *var.pyrocarpa Alef*, 7 — F_7 Yir 5815/Marjan *var.pyrocarpa Alef*, 8 — DH2 F_2 Yir 5815/Marjan *var.pyrocarpa Alef*, 9 — Yir 5815, 10 — Bakanassky, 11 — Pak Lee, 12 — Marjan; Plate 2. M — marker, 13 — standard (IR 04636), 14 — F_8Black Rice/Marjan, 15 — F_8 Black Rice/Marjan *var.pyrocarpa Alef*, 16 — F_8Black Rice/Marjan *var.subpyrocarpa Gust*, 17 — F_8 Black Rice/Bakanassky *var.pseudovialonica Vasc*, 18 — F_8 Black Rice/ Bakanassky *var.Desvauxii Koern*, 19 –- F_8 Black Rice/ Bakanassky *var.Eediana Koern*, 20 — F_8 Black Rice/Bakanassky *var.para-Gastrol Port*, 21 — F_8 Black Rice/Viola *var.Desvauxii Koern*, 22 — DH2 F_2 Black Rice/Bakanassky, 23 — Black Rice, 24 — Marjan, 25 — Bakanassky, 26 — Viola; Plate 3. M — marker, 27 — standard (IR 04636), 28 — F_8 Black Rice/ Yantar anthoc.pigm., 29 — F_8 Black Rice/Yantar *var.nigrispina Port*, 30 — F_8 Mavr/ Kurchanka *var.pyrocarpa Alef*, 31 — F_8 Mavr/ Kurchanka *var.sundensis Koern*, 32 — F_8 Mavr/Pak Lee *var.bansmatica Koern*, 33 — F_8 Mavr/ Bakanassky *var.Desvauxii Koern*, 34 — Black Rice, 35 — Yantar, 36 — Mavr, 37 — Kurchanka, 38 — Pak Lee, 39 — Bakanassky, 40 — Almavita.

Table 2: Molecular screening of initial varieties and lines of rice with stained pericarp for blast resistance genes

Varieties and hybrid lines	Blast resistance genes
Yir 5815	*Pi-40 + Pi-9*
Bakanassky	*Pi-40 + Pi-9 + Pi-54*
Pak Lee	*Pi-40 + Pi-54*
Marjan	*Pi-40 + Pi-9 + Pi-54*
Black Rice	*Pi-40 + Pi-9 + Pi-54*
Viola	*Pi-40 + Pi-9 + Pi-54*
Yantar	*Pi-40 + Pi-9 + Pi-54*
Mavr	*Pi-40 + Pi-9*
Kurchanka	*Pi-40 + Pi-9 + Pi-54*
Almavita	*Pi-40*
F_7 Yir 5815/Bakanassky *var.sundensis* Koern	*Pi-40 + Pi-9*
F_7 Yir 5815/ Bakanassky var.pyrocarpa Alef	*Pi-40 + Pi-9*
F_7 Yir 5815/ Pak Lee var.sundensis Koern	*Pi-40*
F_7 Yir 5815/ Pak Lee var.subpyrocarpa Gust	*Pi-40 + Pi-9*
F_7 Yir 5815/ Pak Lee var.pyrocarpa Alef	*Pi-40 + Pi-9 + Pi-54*
F_7 Yir 5815/Marjan var.pyrocarpa Alef	*Pi-40 + Pi-9*
DH2 F_2 Yir 5815/Marjan var.pyrocarpa Alef	*Pi-40 + Pi-9*
F_8 Black Rice/Marjan	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Marjan *var.pyrocarpa Alef*	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Marjan *var.subpyrocarpa Gust*	*Pi-40 + Pi-54*
F_8 Black Rice/Bakanassky *var.pseudovialonica Vasc*	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Bakanassky *var.Desvauxii Koern*	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/ Bakanassky *var.Eediana Koern*	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Bakanassky *var.para-Gastrol Port*	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Viola *var.Desvauxii Koern*	*Pi-40 + Pi-9 + Pi-54*
DH2 F_2 Black Rice/Bakanassky	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/ Yantar anthoc.pigm.	*Pi-40 + Pi-9 + Pi-54*
F_8 Black Rice/Yantar *var.nigrispina Port*	*Pi-40 + Pi-9 + Pi-54*
F_8 Mavr/ Kurchanka *var.pyrocarpa Alef*	*Pi-40 + Pi-9 + Pi-54*
F_8 Mavr/ Kurchanka *var.sundensis Koern*	*Pi-40 + Pi-9 + Pi-54*
F_8 Mavr/ Pak Lee *var.bansmatica Koern*	*Pi-40 + Pi-9*
F_8 Mavr/ Bakanassky *var.Desvauxii Koern*	*Pi-40 + Pi-9 + Pi-54*

broad-spectrum R genes (Tang et al., 2024). In practice, however, many of these genes are rare or variably effective. For example, Pi-1 genes are quite rare and have been less studied recently, whereas Pi-9 is introgressed from wild *O.minuta* and confers broad resistance but still needs supporting genes to avoid breakdown. The Pi-54 gene is widely regarded as particularly durable and broad-spectrum, and it has been successfully used in recent breeding to improve blast resistance (Nameirakpam et al., 2024). Pi-40 also has broad activity, but as our results indicate, reliance on any single R gene is risky. Notably, we observed that lines carrying only Pi-9 or Pi-40 still suffered some blast, consistent with reports that even "broad-spectrum" genes may lose efficacy unless combined. The results shows that to achieve long term resistance in rice, there is a need to have *Pi-1, Pi-40, Pi-9* and *Pi-54* gene. This aligns with recent studies that are focused on utilizing gene pyramiding as a counter measure for developing blast resistance in rice (Jamaloddin et al., 2020, Ramalingam et al., 2020). Peng et al. (2023) in their study showed that the enhanced genetic lines of blast resistance increased dramatically as compared to Chuang5S, and the three gene pyramiding lines (Pigm + Pi48 + Pi49) showed greater rice blast resistance than the monogenic and digenic lines (Custodio et al., 2019). This aligns with our results and supports the need for gene pyramiding.

Analysis of Yield Structure Elements of Promising Lines of Rice with Colored Pericarp

The elements of yield structure were studied in 16 hybrids, one dihaploid, and the Almavita variety. The main traits were evaluated in points from 1 to 9 according to the methodological guidelines developed by Vavilov (1982).

Tillering is an important trait determining plant productivity. In F_8 *Black Rice/Marjan var.subpyrocarpa Gust* and *DH3* F_2 *Yir 5815/Marjan var.pyrocarpa Alef*, tillering ranges from 2.0±0.5 to 11.0±0.7pcs., respectively, so these lines are characterized by very weak to medium tillering. In the *Almavita* variety, productive tillering reaches 3.0–4.0pcs. Plant height is a significant trait in breeding in terms of resistance to lodging. The dihaploid *DH3* F_2 *Yir 5815/Marjan var.pyrocarpa Alef* has a great level of this trait (115.3±1.5cm) and belongs to the high-growing group, while in the F_7 *Yir 5815/ Marjan var.pyrocarpa Alef* hybrid of the same combination ranks low with 57.2±0.9cm. In the *Almavita* variety, plant height amounts to 65–68cm. The plant height results obtained in this study of Niu et al. (2021) who highlighted that crop height not only determines plant resistance to lodging and crowding, but also affects crop architecture, apical dominance, biomass, and mechanical harvesting (Hasan et al., 2020). Their research also highlighted the importance of lignin in determining crop height.

Panicle length is a persistent genetic trait of plants that correlates with plant height, grain number per panicle, and productivity. According to the classifier of the Council for Mutual Economic Assistance, this attribute is differentiated as follows: less than 10cm – short, 11 – 15cm – medium, 16–25cm – long, more than 25cm – very long. The *dihaploid DH3* F_2 *Yir 5815/Marjan var.pyrocarpa Alef* surpasses all other combinations with its panicle measuring 20.7±0.6cm (very long). On average, this indicator varies from 11.8±0.6cm to 17.1±0.7cm, or from short to medium length, respectively. The panicles of the *Almavita* variety are vertical and medium in length (11–13cm).

The flag leaf plays a major role in light energy absorption and carbon dioxide assimilation. The flat shape of the leaf, which provides a high surface-to-volume ratio, allows for a more complete utilization of sunlight energy (Gromova & Kostylev, 2018). Among the studied lines, the length of the flag leaf varied from 12.5±0.7cm to 31.3±0.9cm, and its width ranged from 0.6±0.05cm to 1.0±0.05cm.

The number of caryopses on the main panicle is one of the key elements in rice productivity. In the studied combinations, this indicator ranged from 43 to 123pcs. The most valuable for breeding are samples with more than 100 caryopses per panicle. The greatest performance by this parameter is demonstrated by F_8 *Black Rice/Viola var.pseudovialonica Vasc* and *DH3 F2 Yir 5815/Marjan var.pyrocarpa Alef* (116.0±2.8 and 123.0±1.2, respectively). In the *Almavita* variety, the number of caryopses per panicle is 83–101pcs.

Lack of grain in plants is greatly affected by abiotic factors. A high level of grain loss is observed in the combinations of F_8 *Mavr/Pak Lee var.bansmatica Koern* and F_8 *Mavr/Bakanassky var.Desvauxii Koern* (17% and 17.1%, respectively). In contrast, the lowest grain loss is found in the F_8 *Black Rice/Bakanassky var.pseudovialonica Vasc* hybrid (1.4%). In the *Almavita* variety, the share of undeveloped caryopses amounts to 3–5%.

According to literary sources, based on stem strength, rice varieties are distinguished into lodging-resistant and lodging-intolerant. Furthermore, there are two different types of lodging: root lodging and stem lodging. The second type of lodging is correlated with weak and brittle straw. On average, this trait ranges from 2.5 mm to 4.0 mm in the studied lines. In the *Almavita* variety, the stem is medium thick, flexible, and resistant to lodging. To determine the seeding rate, it is essential to know the weight of a thousand seeds. This trait can vary greatly within a single variety, depending on growing conditions. The greatest seed weight is observed in the combination of *DH3* F_2 *Yir 5815/Marjan var. pyrocarpa Alef* (38.0±0.2g), while the lowest weight is found in F_8 *Black Rice/Yantar var. nigrispina Port* lines (24.2±0.8g).

Another very important parameter in breeding is awnedness. Awned forms of rice have more vigorous plant development than awnless ones. Because of the struggle against bird damage to rice crops, some rice farms prefer the awned form, yet in many cases the awnless varieties are favored because awn debris causes allergic diseases in humans. Among the lines studied, seven combinations are awnless, six combinations awned, and five combinations semi-awned. The red-grain, glutinous rice variety *Almavita* proposed for State Variety Testing has awnless, anthocyanin-colored, pubescent lemmas.

Thus, in terms of yield, of great interest among the created hybrid lines are F_8 *Black Rice/Viola var.pseudovialonica Vasc* and *DH3* F_2 *Yir 5815/Marjan var.pyrocarpa Alef* with 116.0±2.8 and 123.0±1.2caryopses per panicle, respectively. Great productivity is also demonstrated by *Almavita* with 83–101caryopses per panicle. Importantly, the results and recent literature emphasize that resistance is highly environmentally dependent. Temperature and other factors can modulate R-gene expression and pathogen aggressiveness. Studies show that Pi-54-mediated resistance is reported to be strongest at cool (~22°C) temperatures and weaker at higher temperatures (32°C) (Madhusudhan et al., 2011). Thus, gene effect may not be uniform: a gene that confers strong resistance in one climate or at the seedling stage may fail in another. This environmental specificity highlights a limitation of the current study (conducted under specific field conditions) and of many R-gene trials. It suggests that we must test blast-resistant lines across diverse agroecological zones and seasons to truly assess durability. Furthermore, breeding should focus on stacking multiple R genes with complementary spectra. Recent studies have achieved 3-4 R-gene pyramids that approach complete, broad-spectrum resistance without agronomic penalty. Comparable studies in other pigmented rices indicate that such strategies are feasible (Tang et al., 2024). In parallel, novel sources of partial or adult-plant resistance should be explored, because polygenic partial resistance tends to be more durable and less race-specific. We also recommend closer attention to gene×environment interactions: future work could incorporate controlled-environment trials (e.g., varying temperature or humidity) and correlate R-gene expression with resistance under field weather data. Finally, because colored pericarp rice is valued for its antioxidants and nutritional traits, breeding programs must simultaneously improve blast resistance and maintain grain quality. Our results confirm that achieving durable blast resistance in colored rice will require stacking multiple R genes and validating them across environments; only with such pyramids, rather than reliance on single genes like Pi-40 in Almavita, can broad, durable protection be achieved.

Conclusion

This study applied a marker-assisted selection pipeline based on PCR-SSR genotyping to track blast-resistance loci (*Pi-1, Pi-9, Pi-40, Pi-54*) in colored-pericarp rice backgrounds. The workflow reliably identified target loci with locus-specific markers and validated controls, enabling confident presence/absence calls across late-generation hybrids and varietal samples. Within this set, the variety Almavita was confirmed to carry *Pi-40*, a locus broadly associated with resistance to Magnaporthe oryzae. Field measurements collected under uniform management and summarized as mean ± SD across repeated experiments showed a favorable agronomic profile for Almavita, including high yield potential, robust panicle formation, competitive 1000-grain weight, acceptable lodging scores, and low grain loss. The combination of a strong R-gene background and solid agronomic performance in colored-pericarp material aligns with the program goal of delivering market-oriented germplasm without compromising field productivity. In conclusion, these results support Almavita as a candidate for State Variety Testing and demonstrate the practical utility of the PCR-SSR workflow for advancing colored-pericarp rice with validated Pi-loci in regional breeding.

DECLARATIONS

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Conflict of Interest: There is no conflict of interest.

Data Availability: Data will be available at request.

Ethics Statement: The present study involved plant material (*Oryza sativa* L.) only and did not include experiments on humans or animals. All field and laboratory procedures were carried out in accordance with the institutional guidelines and research regulations of the Institute of Plant Biology and Biotechnology, Republic of Kazakhstan, as well as relevant national standards. Ethical approval was not required for this study, as no activities involving human participants or animals were conducted.

Author's Contribution: Conceptualization, experimental design and original draft preparation of manuscript – A.A. and G.Z.; supervision – B.U., interpretation and editing – Y.G., Sh.A. and B.A.; conducting experiments – I.S., Sh.Y., S.T. and M.N. All authors have read and agreed to the published version of the manuscript.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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