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Development of rice lines by pyramiding of major blast resistance genes

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Keywords: Rice; hybrid; Magnaporthe oryzae; resistance; Marker Assisted Selection (MAS); gene pyramiding; phytopathology

Article

Enhancing Rice Blast Resistance by Gene Pyramiding and MAS Analysis

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Abstract: Rice blast is the most harmful disease caused by *Magnaporthe oryzae*, leading to yield loss. The aim of the work is to improve the resistance of rice to blast by introducing pyramid genes using conventional breeding and modern MAS techniques. Pyramiding is the combination of several resistance genes in one genotype, which improves the resistance of rice to pathogens. In this study, 7 pyramidal lines were identified with the introduction of a combination of 2 blast resistance genes (*Pi-1* and *Pi-ta*) into 6 hybrids and 3 genes (*Pi-1*, *Pi-33* and *Pi-ta*) into 1 line. Phytopathological testing of local rice cultivars and pyramidal lines showed that 3 out of 7 pyramidal lines were moderate resistant: 2 lines (F_2 Bakanasski/7667 *var. vulgaris* and F_2 Aisaule/7664 *var. italica*), containing 2 resistance genes (*Pi-1* and *Pi-ta*) and 1 line – F_2 Aisaule/7689 *var. zeravschanica*, carrying 3 genes (*Pi-1*, *Pi-33* and *Pi-ta*), while the local cultivars Bakanasski, Aisaule and Aru, containing the blast resistance gene *Pi-2*, are susceptible. As a result, the blast resistance of rice was improved by creating pyramidal lines. Pyramided lines can be used for germplasm exchange and in rice breeding programs to improve blast resistance in rice.

Keywords: rice; hybrid; *Magnaporthe oryzae*; resistance; Marker Assisted Selection (MAS); gene pyramiding; phytopathology

1. Introduction

Rice blast is one of the most dangerous diseases [1,2], and the causative agent of this disease is the parasitic fungus *Magnaporthe oryzae*. Defeat of rice by pathogen in the early stages of development leads to a decrease in seed germination, sparseness of crops and death of seedlings. The greatest harm from infection occurs during the heading and flowering, which is associated with the formation of underdeveloped or feeble seeds, which significantly reduces the quality of the grain [3]. Blast reduces rice production in the world, causing crop losses from 15-40% and reaching up to 80-100% in epiphytotic years [4].

Global climate change affects the evolution of pathogen biotypes, which challenges breeders and forces them to increase rice productivity by creating and introducing new cultivars resistant to changing races or isolates of *Magnaporthe oryzae* [5]. Currently, more than 100 blast resistance genes have been discovered [5–15]. Most of them with broad spectrum resistance to pathogen [5,6,16–19], there are some major genes with a wide spectrum of resistance, such as: *Pi-z5* (*Pi-2*) [20], *Pi-9* (*t*) [21], *Pigm* [22–24], *Pi-ta* [25], *Pi-1* and *Pi-33* [7–14,26], which serve as indicators of blast resistance gene clusters, which is important when choosing parental pairs for hybridization. Most blast resistance genes are dominant, some of them are quantitative [27], and most of them, such as *Pita*, *Pi-1*, *Pi25*, *Pigm* and *Pia*, encode proteins that recognize the signal and inhibit the growth of the phytopathogen, maintaining plant immunity [23,28–30]. For example, the *Pita* gene encodes a cytoplasmic membrane

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receptor protein that leads to resistance. [31]. And the *Pigm* gene encodes two proteins, PigmR and PigmS, which regulate resistance and yield of rice plants [23]. Recently, the *Pigm-1* gene has been found, encoding the Pigm-1 protein, which is an allelic variant of PigmR [32]. Moreover, many resistance genes are clustered on chromosomes 6, 9, 11, and 12 [33,34]. Disease resistance is controlled by one or two [35,36], three [37] or more pairs of genes [38]. Typically, rice has one or two dominant resistance genes that are effective against one race of the fungus [39].

Determination of resistance genes in genetically diverse rice material is important to identify new sources of blast resistance. DNA markers are used to identify resistance genes [26]. The most effective way to combat blast is to grow resistant rice cultivars. However, after a few years, the cultivar loses resistance due to the high variability of rice pathogens [40,41].

The selection of disease-resistant cultivars based on conventional breeding methods is complicated due to the difficulty of determining the presence of the desired allele of a particular gene. The use of molecular markers closely linked to genes that provide resistance to the pathogen greatly facilitates breeding work [42]. Therefore, the main factor in rice breeding for blast resistance is the selection of resistance donors based on the identification of genes that control this trait in rice and the creation of new cultivars with a high level of resistance [43]. Success in combat with rice blast has been achieved by introducing and pyramiding major blast resistance genes using MAS [6–14]. Most resistance genes provide rice immunity only to certain races of the pathogen. It is known that such genes, when pyramided in one genotype, can provide stable resistance to the disease.

Pyramiding of multiple resistance genes in a single genotype is the most efficient strategy in breeding for resistance to a variable population of *Magnaporthe oryzae*. Cultivars that combine combinations of 3-5 resistance genes are more resistant, as they show an increase and expansion of the spectrum of resistance to blast. Thus, the introduction of genes by combining (pyramiding) several blast resistance genes in one genotype is the most promising direction of rice selection [7–14,43]. Screening cultivars for the presence of genes for resistance to *Magnaporthe oryzae*, as well as the introduction and pyramid of resistance genes to the pathogen, contribute to a significant reduction in the time for rice improvement. Introduction of resistance genes into local cultivars is a preferred strategy in a rice breeding program for disease prevention. The aim of the work is to improve the resistance of rice to *Magnaporthe oryzae* based on the introduction and pyramid of the major blast resistance genes using MAS.

2. Results

2.1. Screening of Parental Rice Genotypes for Blast Resistance Genes Pi-1 and Pi-2

According to the electropherogram, the presence of the *Pi-1* gene was identified in 4 samples 04636 (well 5), 03-27 (well 6), 04468 (well 7), 04470 (well 8) and they are heterozygous, 7668 (well 19) homozygous for *Pi-1* because it repeated the DNA profile of the positive control C101-Lac (Figure 1A). Several samples (03-27 (well 6), 04468 (well 7), 04470 (well 8) and 7662 (well 14) homozygous for the *Pi-2* gene were found, since they are identical in profile to the DNA of the differentiator cultivar C101-A51, the carrier *Pi-2* gene (Figure 1A). The sizes of PCR products corresponding to the dominant genes *Pi-1* and *Pi-2* are 133 bp and 325 bp, respectively.

In Figure 1B shows that samples 7679 (well 5), 7683 (well 6), 7684 (well 7), 7689 (well 8) and 7695 (well 10) turned out to be homozygous for the Rm224 and Rm144 loci, which indicates the presence of a dominant rice blast resistance gene *Pi-1*. The following samples 7702 (well 12) and 7712 (well 13) are heterozygous for this gene. At the Rm527 and SSR140 loci, samples 7684 (well 7), 7690 (well 9), Bakansski (well 16), Aisaule (well 18) and Aru (well 19) showed DNA profiles like the positive control C101-A51 for the *Pi-2* gene. In the remaining samples, the presence of genes in their genotypes in recessive states was revealed.

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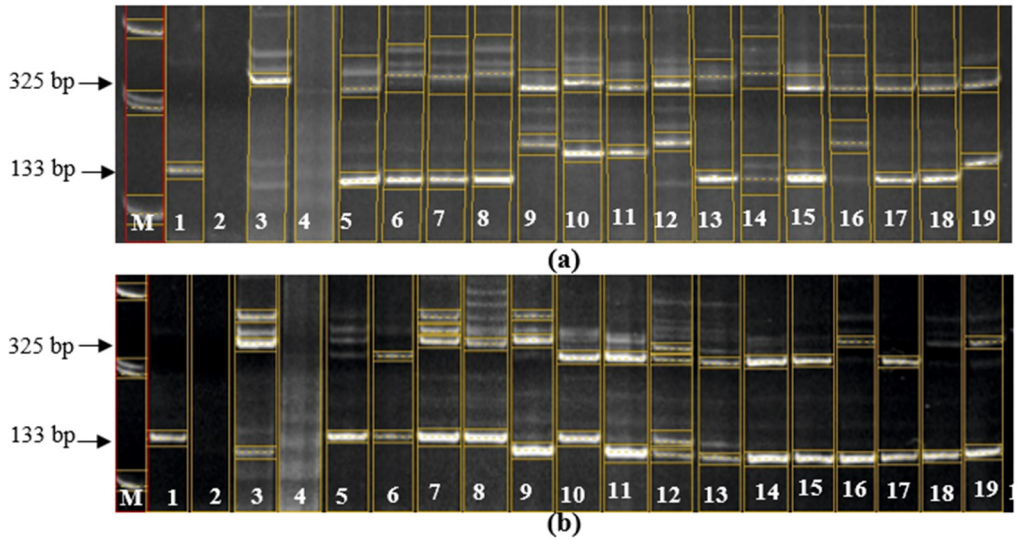

Figure 1. Electropherogram of PCR products of samples for the presence of the *Pi-1* and *Pi-2* genes. (**a**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-1* gene at the Rm144 and RM224 loci), 2 – Flagman (control "-" for the *Pi-1* gene at the Rm144 and RM224 loci), 3 – C101-A51 (control "+" for the *Pi-2* gene at loci RM-527 and SSR-140), 4 – Flagman (control "-" for the *Pi-2* gene at loci RM-527 and SSR-140), 5 – 04636, 6 – 03-27, 7 – 04468, 8 – 04470, 9 – 04469, 10 – 25-14, 11 – 04888, 12 – 212-05, 13 – 7653, 14 – 7662, 15 – 7663, 16 – 7664, 17 – 7666, 18 – 7667, 19 – 7668; (**b**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-1* gene at the Rm144 and RM224 loci), 2 – Flagman (control "-" for the *Pi-1* gene at the Rm144 and RM224 loci), 3 – C101-A51 (control "+" for the *Pi-2* gene at loci RM-527 and SSR-140), 4 – Flagman (control "-" for the *Pi-2* gene at loci RM-527 and SSR-140), 5 – 7679, 6 – 7683, 7 – 7684, 8 – 7689, 9 – 7690, 10 – 7695, 11 – 7701, 12 – 7702, 13 – 7712, 14 – 7824, 15 – Don 7712, 16 – Bakanasski, 17 – Fatima, 18 – Aisaule, 19 – Aru.

2.2. Screening of Parental Genotypes for the Presence of Pi-33 and Pi-ta Genes

The electropherogram of Figure 2A shows that samples 7662 (well 16) and 7663 (well 17) were identified as carriers of a homozygous dominant allele of the resistance gene Pi-33, since these genotypes repeat the resistance gene carrier profile of cv. C101-Lac. The heterozygous state of the Pi-ta gene was found in genotypes 03-27 (well 8) and 04468 (well 9). Also genotypes 25-14 (well 12) and 7664 (well 18) were homozygous for this locus (Figure 2A), because these genotypes match the DNA pattern of the positive control IR36. The sizes of PCR products corresponding to the dominant genes Pi-33 and Pi-ta are 198 bp and 400 bp, and for recessive genes are 300 bp and 600 bp, respectively. According to the results (Figure 2B), the Pi-33 resistance gene was found in a homozygous state in genotypes 7668 (well 6), 7679 (well 7), 7684 (well 9), 7686 (well 10), 7689 (well 11) and 7690 (well 12) compared to C101-Lac. The locus co-segregating with the dominant resistance gene Pi-ta was found in genotypes 7686 (well 10) and 7701 (well 15), (Figure 2B), while in genotypes 7667 (well 5) the Pi-ta gene is in a heterozygous state. The genotype 7686 (well 10) repeats the bands of two positive controls IR36 and C101-Lac, since it carries both Pi-33 and Pi-ta genes. Figure 2C shows that local rice cultivars did not have Pi-33 and Pi-ta resistance genes in their genotypes.

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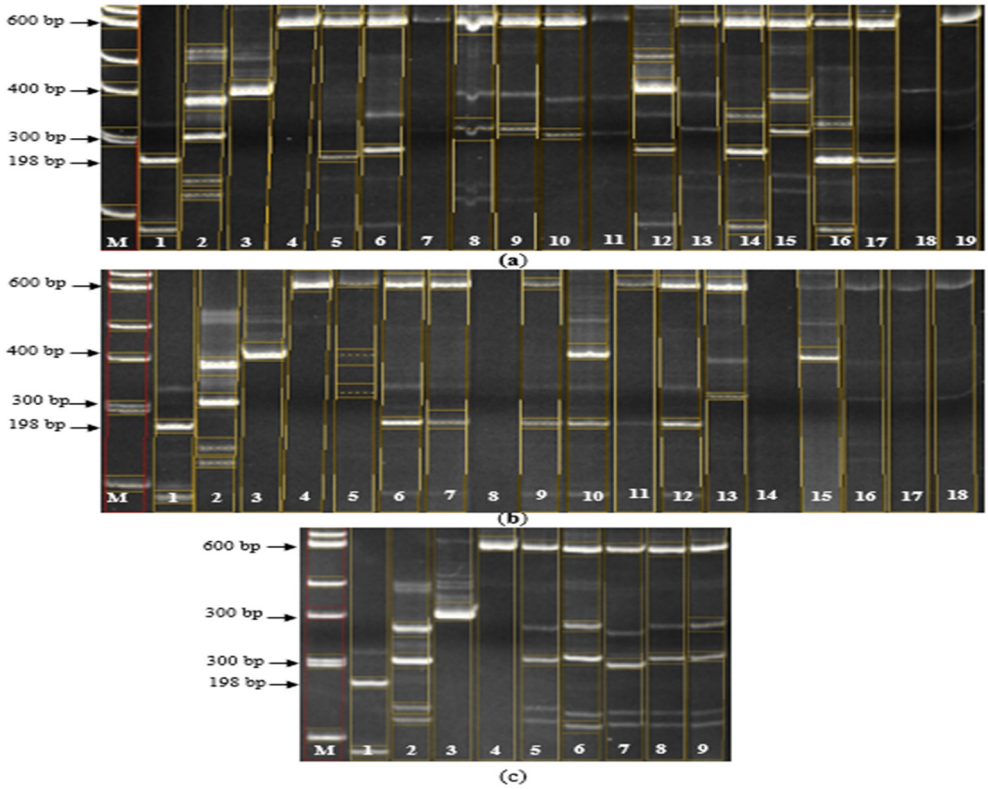

Figure 2. Electropherogram of PCR products of samples for the presence of *Pi-33* and *Pi-ta* genes. (**a**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci); 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci); 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci); 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci); 5 – 04636; 6 – 19-14; 7 – 57-14; 8 – 03-27; 9 – 04468; 10 – 04470; 11 – 04469; 12 – 25-14; 13 – 04888; 14 – 212-05; 15 – 7653; 16 – 7662; 17 – 7663; 18 – 7664; 19 – 7666; (**b**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR 36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – 7667, 6 – 7668, 7 – 7679, 8 – 7683, 9 – 7684, 10 – 7686, 11 – 7689, 12 – 7690, 13 – 7695, 14 – 7698, 15 – 7701, 16 – 7702, 17 – 7712, 18 – 7824; (**c**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – Don 7712, 6 – Bakanasski, 7 – Fatima, 8 - Aisaule, 9 – Aru.

According to electropherogram data from 4 analyzed local high-yielding cultivars used as maternal lines in crossings, it turned out that three cultivars Bakanasski, Aisaule and Aru contain the *Pi-2* gene. It should be noted that all 4 local genotypes do not contain the other 3 studied genes (*Pi-1*, *Pi-33* and *Pi-ta*). Therefore, further experiments were conducted to introduce and pyramid these genes into local cultivars using hybridization and MAS techniques to improve rice blast resistance.

2.3. Screening of Rice Hybrids for the Presence of Pi-1 and Pi-2 Genes

Figure 2C shows that local rice cultivars do not have *Pi-33* and *Pi-ta* resistance genes in their genotypes shows that 4 hybrids, 7824/Aisaule *var. italica* (well 11), 7824/Aisaule *var. subuzbekistanica* (well 12), 7698/Bakanasski *var. subvulgaris* (well 14), 7698/Bakanasski *var. italica* (well 15) are heterozygotes for *Pi-2 gene (*Figure 3A). The sizes of PCR products corresponding to the dominant genes *Pi-1* and *Pi-2* are 133 bp and 325 bp, for recessive genes are 120 bp 313 bp, respectively. The following 13 hybrids were found to contain *Pi-1* gene in homozygous state in their genotypes: 7698/Aisaule *var. suberythroseros* (well 5), Aru/7702 *var. erythroceros* (well 6), Aisaule/7689 *var. zeravschanica* (well 7), Aru/0327 *var. vulgaris* (well 8), Aru/0327 *var. subpyrocarpa* (well 9),

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Bakanasski/7667 *var. subjanthoseros* (well 10), Bakanasski/7667 *var. vulgaris* (well 11), Bakanasski/7701 *var. vulgaris* (well 12), Aisaule/7664 *var. italica* (well 13), Aisaule/04470 *var. subvulgaris* (well 15), Aisaule/04470 *var. italica* (well 16), Aisaule/Don 7712 *var. sundensis* (well 17), Aisaule/Don 7712 *var. italica* (well 18) (Figure 3B).

The DNA profiles of the above hybrid lines were like those of differentiator cultivars C101-Lac and C101-A51, indicating successful transfer of blast resistance genes.

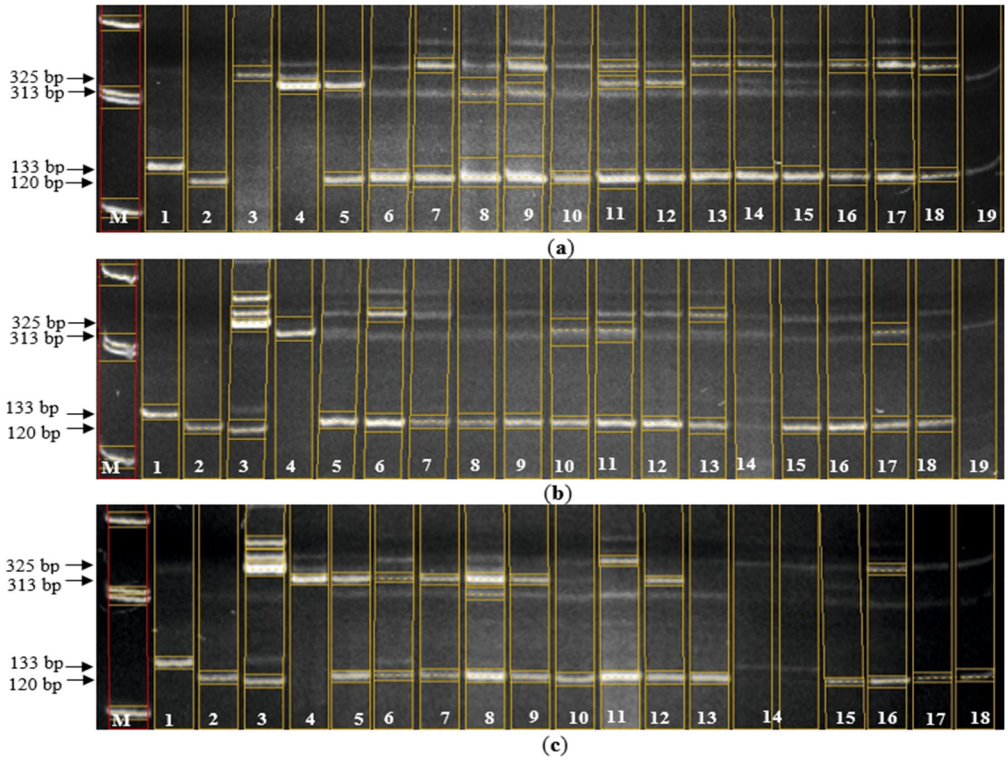

Figure 3. Electropherogram of PCR products of hybrids for the presence of *Pi-1* and *Pi-2* genes. (**a**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-1* gene at the Rm144 and RM224 loci), 2 – Flagman (control "-" for the *Pi-1* gene at the Rm144 and RM224 loci), 3 – C101-A51(control "+" for the *Pi-2* gene at loci RM-527 and SSR-140), 4 – Flagman (control "-" for the *Pi-2* gene at loci RM-527 and SSR-140), 5 – Bakanasski/7668 *var. subuzbekistanica*, 6 – Bakanasski/04470 *var. vulgaris*, 7 – Bakanasski/7653 *var. subuzbekistanica*, 8 – Bakanasski/7653 *var. subjanthoseros*, 9 – Bakanasski/7684 *var. subvulgaris*, 10 – Bakanasski/7684 *var. vulgaris*, 11 – 7824/Aisaule *var. italica*, 12 – 7824/Aisaule *var. subuzbekistanica*, 13 – Aru/04468 *var. subvulgaris*, 14 – 7698/Bakanasski *var. subvulgaris*, 15 – 7698/Bakanasski *var. italica*, 16 – Aru/7701 *var. vulgaris*, 17 – Bakanasski/0327 *var. vulgaris*, 18 – Aisaule/7679 *var. subvulgaris*, 19 –7698/Aisaule *var. italica* (**b**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-1* gene at the Rm144 and RM224 loci), 2 – Flagman (control "-" for the *Pi-1* gene at the Rm144 and RM224 loci), 3 – C101-A51(control "+" for the *Pi-2* gene at loci RM-527 and SSR-140), 4 – Flagman (control "-" for the *Pi-2* gene at loci RM-527 and SSR-140), 5 – 7698/Aisaule *var. suberythroseros*, 6 – Aru/7702 *var. erythroceros*, 7 – Aisaule/7689 *var. zeravschanica*, 8 – Aru/0327 *var. vulgaris*, 9 – Aru/0327 *var. subpyrocarpa*, 10 – Bakanasski/7667 *var. subjanthoseros*, 11 – Bakanasski/7667 *var. vulgaris*, 12 – Bakanasski/7701 *var. vulgaris*, 13 – Aisaule/7664 *var. italica*, 14 – Aisaule/7664 *var. subvulgaris*, 15 – Aisaule/04470 *var. subvulgaris*, 16 – Aisaule/04470 *var. italica*, 17 – Aisaule/Don 7712 *var. sundensis*, 18 – Aisaule/Don 7712 *var. italica*, 19 – Fatima/7695 *var. italica* (**c**): M – Molecular marker 100 bp; 1 – C101-Lac (control "+" for the *Pi-1* gene at the Rm144 and RM224 loci), 2 – Flagman (control "-" for the *Pi-1* gene at the Rm144 and RM224 loci), 3 – C101-A51 (control "+" for the *Pi-2* gene at loci RM-527 and SSR-140), 4 – Flagman (control "-" for the *Pi-2* gene at loci RM-527 and SSR-140), 5 – Fatima/7695 *var. breviaristata*, 6 – Bakanasski/7683 *var. subjanthoseros*, 7 – 7698/Aru *var. erythroceros*, 8 – 7698/Aru *var. suberythroseros*, 9 – Bakanasski/Don 7712, 10 – Bakanasski/Don 7712 *var. Desvauxii*, 11 – Aru/212-05 *var. vulgaris*, 12 – Aru/7668 *var. vulgaris*, 13 – Aisaule/04468 *var. italica*, 14 – Aru/04636

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var. vulgaris, 15 – Bakanasski/7690 *var. subjanthoseros*, 16 – Bakanasski/7690 *var. vulgaris*, 17 – Bakanasski/04468 *var. subvulgaris*, 18 – Bakanasski/04468 *var. vulgaris.*

The electropherogram of Figure 3C demonstrates the heterozygous state of the hybrid Bakanasski/7683 *var. subjanthoseros* (well 6) for the *Pi-1*. The hybrid line Aru/04636 var. vulgaris was found to be dominant homozygote for this gene (well 14).

3.4. Screening of Rice Hybrids for the Presence of Pi-33 and Pi-ta Genes

Figure 4A shows that the hybrid Bakanasski/7684 *var. subvulgaris* (well 9) has the *Pi-33* target gene in its genotype in a homozygous state, and the line Bakanasski/7668 *var. subuzbekistanica* (well 5) contains the dominant *Pi-ta* resistance gene. The sizes of PCR products corresponding to the dominant genes *Pi-33* and *Pi-ta* are 198 bp and 400 bp, and for recessive genes are 300 bp and 600 bp, respectively.

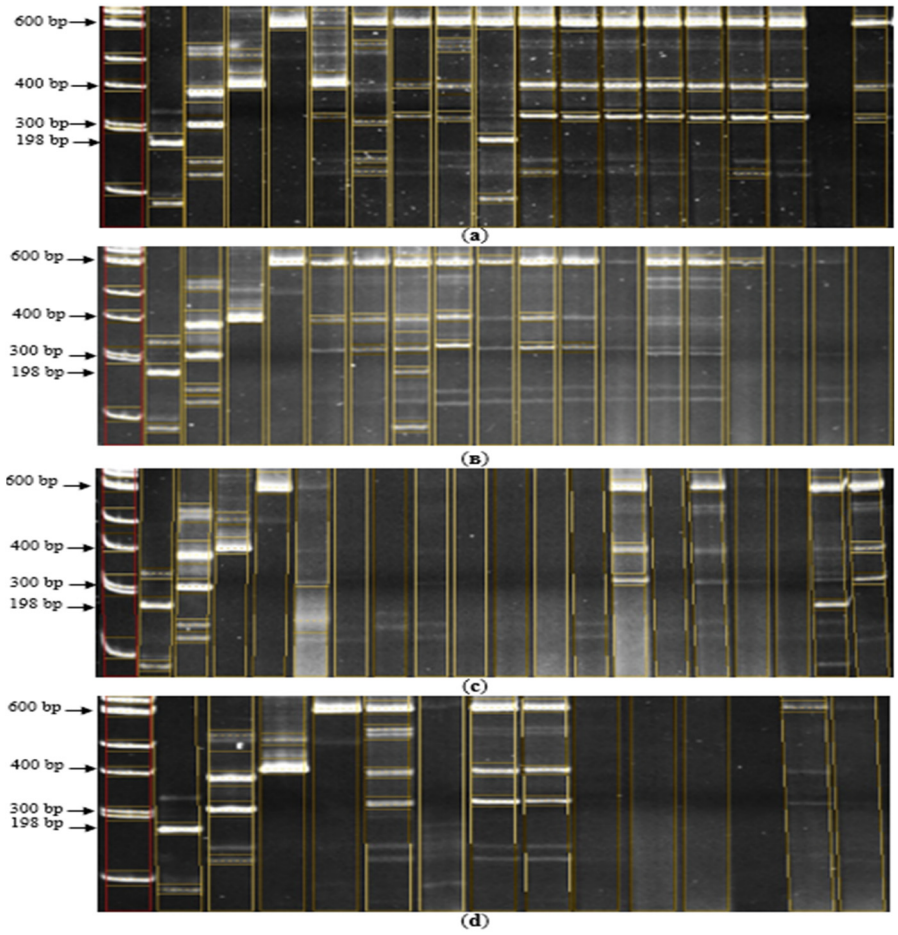

Figure 4. Electropherogram of PCR products of hybrids for the presence of *Pi-33* and *Pi-ta* genes. (**a**): M – Molecular marker 100 bp; 1 - C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – Bakanasski/7668 *var. subuzbekistanica*, 6 – Bakanasski/04470 *var. vulgaris*, 7 – Bakanasski/7653 *var. subuzbekistanica*, 8 – Bakanasski/7653 *var. subjanthoseros*, 9 – Bakanasski/7684 *var. subvulgaris*, 10 – Bakanasski/7684 *var. vulgaris*, 11 – 7824/Aisaule *var. italica*, 12 – 7824/Aisaule *var. subuzbekistanica*, 13 – 7698/Bakanasski *var. subvulgaris*, 14 – 7698/Bakanasski *var. italica*, 15 – Aru/7701 *var. vulgaris*, 16 – Bakanasski/0327 *var. vulgaris*, 17 – Aisaule/7679 *var. subvulgaris*, 18 – 7698/Aisaule *var. italica* (**в**): M – Molecular marker 100 bp; 1 - C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – 7698/Aisaule *var. suberythroseros*, 6 – Aru/7702 *var. erythroceros*, 7 – Aisaule/7689 *var. zeravschanica*, 8 – Aru/0327 *var.*

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vulgaris, 9 – Bakanasski/7667 *var. vulgaris*, 10 – Bakanasski/7701 *var. vulgaris*, 11 – Aisaule/7664 *var. italica*, 12 – Aisaule/7664 *var. subvulgaris*, 13 – Aisaule/04470 *var. subvulgaris*, 14 – Aisaule/04470 *var. italica*, 15 – Aisaule/Don 7712 *var. sundensis*, 16 – Aisaule/Don 7712 *var. italica*, 17 – Fatima/7695 *var. breviaristata*, 18 – Bakanasski/7683 *var. subjanthoseros* (**c**): M – Molecular marker 100 bp; 1 - C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – Aru/212-05 *var. vulgaris*, 6 – Aru/7668 *var. vulgaris*, 7 – Aisaule/04468 *var. italica*, 8 – Aru/04636 *var. vulgaris*, 9 – Bakanasski/7690 *var. subjanthoseros*, 10 – Bakanasski/7690 *var. vulgaris*, 11 – Bakanasski/04468 *var. subvulgaris*, 12 – Bakanasski/04468 *var. vulgaris*, 13 – Aru/7666 *var. suberythroseros*, 14 – Fatima/7653 *var. persica*, 15 – Aru/Don 7712 *var. Desvauxii semi-awned*, 16 – Aru/7683 *var. janthoceros*, 17 – Aru/7683 *var. vulgaris*, 18 – Aisaule/7663 *var. italica*, 19 – Aru/0327 *var. subpyrocarpa* (**d**): M – Molecular marker 100 bp; 1 - C101-Lac (control "+" for the *Pi-33* gene at the Rm310 and RM72 loci), 2 – Flagman (control "-" for the *Pi-33* gene at the Rm310 and RM72 loci), 3 – IR36 (control "+" for the *Pi-ta* gene at the Pita loci), 4 – Flagman (control "-" for the *Pi-ta* gene at the Pita loci), 5 – Bakanasski/Don 7712, 6 – Fatima/7689 *var. italica*, 7 – Aru/04468 *var. subvulgaris*, 8 – Bakanasski/7667 *var. subjanthoseros*, 9 – Bakanasski/Don 7712 *var. Desvauxii*, 10 – Aru/*Don 7712 var. vulgaris*, 11 – 7698/Aru *var. erythroceros*, 12 – 7698/Aru *var. suberythroseros*, 13 – Aru/Don 7712 *var. erythroceros*, 14 – Aru/Don 7712 *var. Desvauxii.*

The electropherogram in Figure 4B showed that the hybrid line Aisaule/7689 *var. zeravschanica* (well 7) is heterozygous for the *Pi-33* resistance gene. Figure 4B demonstrates the heterozygous state of the *Pi-ta* allele found in 7 hybrid lines: 7698/Aisaule *var. suberythroseros* (well 5), Aru/7702 *var. erythroceros* (well 6), Aisaule/7689 *var. zeravschanica* (well 7), Aru/0327 *var. vulgaris* (well 8), Bakanasski/7667 *var. vulgaris* (well 9), Bakanasski/7701 *var. vulgaris* (well 10) and Aisaule/7664 *var. italica* (well 11). Electropherogram Figure 4C demonstrates that the line Aisaule/7663 *var. italica* (well 18) is heterozygous for the *Pi-33* gene. The following lines Aru/7666 *var. suberythroseros* (well 13), Aru/Don 7712 *var. Desvauxii* semi-awned (well 15), and Aru/0327 *var. subpyrocarpa* (well 19) are heterozygous for the *Pi-ta* blast resistance gene.

The last electropherogram Figure 4D shows that the lines Bakanasski/Don 7712 (well 5), Aru/04468 *var. subvulgaris* (well 7), Bakanasski/7667 *var. subjanthoseros* (well 8) and Aru/Don 7712 *var. erythroceros* (well 13) have a heterozygous state of the *Pi-ta* allele in their genotypes. Consequently, on electrophoresis of PCR products of the *Pi-33* and *Pi-ta* genes, the presence of both homozygous and heterozygous lines is observed in the hybrid offspring. All other analyzed samples showed DNA profile compliance with the negative control cultivar Flagman, which indicates the presence of a recessive allele of the *Pi-33* and *Pi-ta* genes, respectively.

According to electropherograms of PCR products, 28 hybrid lines were identified whose genotypes contain genes for resistance to blast disease. It was revealed that 7 hybrids of them are pyramidal: 6 hybrids 7698/Aisaule *var. suberythroseros*, Aru/7702 *var. erythroceros*, Bakanasski/7667 *var. subjanthoseros*, Bakanasski/7667 *var. vulgaris*, Bakanasski/7701 *var. vulgaris*, Aisaule/7664 *var. italica* contain 2 blast resistance genes– *Pi-1* and *Pi-ta, and* the hybrid Aisaule/7689 *var. zeravschanica* 3 genes are pyramided – *Pi-1*, *Pi-33* and *Pi-ta*. The remaining hybrids have one of the studied genes each.

2.5. Phytopathological Test of Cultivars and Pyramided Lines for Resistance to Blast Disease

Phytopathological testing of local cultivars used as maternal lines showed that the cultivars Bakanasski, Aisaule and Aru are susceptible, and the cultivar Fatima is moderately resistant. Testing of the resistance of hybrid lines allowed us to identify level of resistance of hybrids to the phytopathogen. Figure 5 shows the reaction to disease of resistant control, susceptible control and improved pyramidal rice lines characterized as moderately resistant.

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Figure 5. Degree of leaf resistance to blast: (**a**) resistant, (**b**) susceptible, (**c**) moderately resistant.

Among the seven pyramided lines selected, one line failed to grow under infectious nursery conditions. In results, of the remaining 6 pyramided lines, 2 lines - Bakanasski/7667 *var. vulgaris* and Aisaule/7664 *var. italica* (carrying 2 resistance genes *Pi-1* and *Pi-ta*) and 1 line – Aisaule/7689 *var. zeravschanica* (carrying 3 genes – *Pi-1*, *Pi-33* and *Pi-ta*) showed moderate resistance (MR) were developed (Table 1).

Table 1. Phytopathological assessment of blast resistance of obtained pyramidal lines and local rice cultivars.

Hybrid lines	**Blast resistance**			
	Intensity of disease development, %	**Degree of resistance to disease**	**Resistance genes**	**Line's reaction**
Bakanasski	55.6	susceptible	*Pi-2*	S
Aisaule	55.6	susceptible	*Pi-2*	S
Aru	66.7	susceptible	*Pi-2*	S
Fatima	44.4	moderately resistant	-	MR
7698/Aisaule *var. suberythroseros*	-	-	-	This line didn't grow up
Aru/7702 *var. erythroceros*	61.1	susceptible	*Pi-1*, *Pi-ta*	S
Aisaule/7689 *var. zeravschanica*	36.7	moderately resistant	*Pi-1*, *Pi-33*, *Pi-ta*	MR
Bakanasski/7667 *var. subjanthoseros*	61.1	susceptible	*Pi-1*, *Pi-ta*	S
Bakanasski/7667 *var. vulgaris*	38.9	moderately resistant	*Pi-1*, *Pi-ta*	MR

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Bakanasski/7701 *var. vulgaris*	72.2	susceptible	*Pi-1*, *Pi-ta*	S
Aisaule/7664 *var. italica*	44.4	moderately resistant	*Pi-1*, *Pi-ta*	MR

MR – moderately resistant, S – susceptible.

2.6. Assessment of the Productivity of Pyramided Rice Lines

The evaluation of the productivity created 6 lines of rice from 7 selected hybrid lines (1 line not grown under the infectious diseases nursery) (Aru/7702 *var. erythroceros*, Bakanasski/7667 *var. subjanthoseros*, Bakanasski/7667 *var. vulgaris*, Bakanasski/7701 *var. vulgaris*, Aisaule/7664 *var. italica*, Aisaule/7689 *var. zeravschanica*) performed by analysis of yield structure elements. The lines were evaluated based on main productivity trait, such as "weight of 1000 seeds, g." Analysis of variance showed that pyramidal lines were inferior to the standard in yield characterization, since there was significant difference between the groups, p-value < 0.001 (Table 2).

Table 2. Analyses of the productivity of pyramided rice lines by One-way ANOVA.

	SS	*df*	*MS*	*F*	*P-value*	*F crit*
Between	367.12	6	61.19*	27.07	0.00	3.25
Within	97.18	43	2.26			

According to the characteristic of yield productivity pyramidal lines are lower in comparison with the control cultivar Bakanasski, which is highly productive for Almaty region.

3. Discussion

There are most of the research works focused on the creation of pyramidal rice lines carrying two or more blast resistance genes using MAS and hybridization methods. MAS analysis facilitates the identification of genes, their introduction and pyramiding into new lines [44–46], that significantly speeds up rice breeding. The main goal of rice breeders in combating blast is to develop new cultivars and lines with broad-spectrum and durable resistance [7–14,43]. Rice lines carrying only one major resistance gene cannot withstand a changing population of the fungus [8]. In this case, the development of cultivars carrying not only one gene, but the pyramid of several genes of resistance to blast will help overcome this disease [47–49].

However, there is some research on assessing the resistance of created lines to phytopathogens in a natural or artificial infectious background which identify the actual degree of resistance of newly created lines. Jiang, H. et al. [8] developed several lines containing two blast resistance genes (*Pi 37*/*Pid 3*), (*Pi 5*/*Pi54*), (*Pi 54*/*Pid3*) and (*Pigm*/*Pid 37*), which showed high resistance to leaf and neck blast under natural infection conditions. Dubina et al. created pyramidal rice lines carrying five blast resistance genes (*Pi-1*, *Pi-2*, *Pi-33*, *Pi-ta* and *Pi-b*) in a homozygous state, and obtained cultivars (Pentagen, Magnate, Pirouette, Argamac, Kapitan, Lenaris) [48]. Kostylev, P.I. et al. [49] obtained pyramided rice lines with 2–6 genes of resistance to blast (*Pi-1*, *Pi-2*, *Pi-33*, *Pi-ta*, *Pi-b* and *Pi-40*). Evaluation of the resistance of pyramided lines to fungal infection showed that lines containing 2 or more genes are more resistant compared to monogenic lines. Ji, Z.J. et al. [50] created a pyramidal line with the *Pi1* and *Pi2* genes, which demonstrated resistance to blast. Positive results obtained by different researchers confirm the effectiveness of using these gene combinations (*Pi-1*, *Pi-2*, *Pi-33* and *Pi-ta*) in improving rice protection against blast. The combination of several genes in one genotype has shown the greatest efficiency in the strategy of durable resistance of rice to blast [7–14,43].

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However, there is evidence that rice lines carrying both individual resistance genes [44,51] and lines with pyramidal genes can have a broad spectrum of resistance to *Magnaporthe oryzae* [45,52]. This mainly depends on weather conditions and the degree of pathogenicity of the fungal strains.

It is well known that resistance of rice cultivars is enhanced by pyramiding of several resistance genes [51]. Wang, X. et al. [52] reported the creation of 74 lines by introgression and pyramiding of genes for resistance to biotic factors such as leafhopper (BPH), blast, fire blight (BLB) and aroma gene. These improved rice lines (ILs), containing combinations of 1 to 4 resistance genes to BPH, blast, BLB and aroma gene, were characterized by moderate or high resistance to multiple biotic stresses [52].

It should be noted that the introduction and pyramiding of several genes in lines sometimes does not lead to an increase in the resistance of the lines and can even sometimes have a negative effect. The introduction of several genes into one line, they may be incompatible, suppressing each other's action and leading to sensitivity to the phytopathogen or to a decrease in the yield of the lines [53].

The results of molecular screening of parental samples for the presence of genes showed that locally zoned varieties contain one of the four resistance genes studied – the *Pi-2* gene, which is present in only three varieties (Bakanasski, Aisaule and Aru). Introgression of blast resistance genes into local zoned cultivars from differentiation varieties was successful. Seven pyramidal lines of rice are created. Three of them were found to show improved resistance to blast according to the results of the phytopathological test: 2 lines - F_2 Bakanasski /7667 *var. vulgaris* and F_2 Aisaule /7664 *var. italica*, containing 2 resistance genes (*Pi-1* and *Pi-ta*) and 1-line F_2 Aisaule /7689 *var. zeravschanica*, carrying 3 genes (*Pi-1*, *Pi-33* and *Pi-ta*).

The resistance of pyramidal lines was assessed under infectious nursery conditions, which made it possible to identify the level of resistance of the created lines. The results of the phytopathological testing demonstrated that these three pyramidal lines with pyramidal 2 and 3 resistance genes showed moderately resistance to a mixture of fungal conidia of different isolates of *M. oryzae*, while 3 local varieties (Bakanasski, Aisaule and Aru) containing 1 resistance gene (*Pi-2*) – sensitive to phytopathogen. Thus, disease resistance of rice was enhanced by creating pyramidal lines. The test results showed that half of the pyramidal lines created were moderately resistant, and the other half were sensitive. This indicates that the level of resistance of the lines is revealed when encountering a phytopathogen in the conditions of an infectious nursery.

4. Materials and Methods

4.1. Plant Materials

The 35 genotypes of local and foreign selection from the rice collection of the Institute of Plant Biology and Biotechnology (IPBB, Almaty, Kazakhstan): 04636, 19-14, 57-14, 03-27, 04468, 04470, 04469, 95-06, 25-14, 04888, 212-05, 7653, 7662, 7663, 7664, 7666, 7667, 7668, 7679, 7683, 7684, 7686, 7689, 7690, 7695, 7698, 7701, 7702, 7712, 7824, Don 7712, Bakanasski, Fatima, Aisaule, Aru were used in the crossing as parents. 54 hybrid lines of the F_2 generation (Bakanasski/7668 *var. subuzbekistanica Kond.*, Bakanasski / 04470 *var. vulgaris Koern.*, Bakanasski/7653 *var. subuzbekistanica Kond.*, Bakanasski/7653 *var. subjanthoseros Brsch.*, Bakanasski/7684 *var. subvulgaris Brsch.*, Bakanasski/7684 *var. vulgaris Koern.*, 7824/Aisaule *var. italica Alef.*, 7824/Aisaule var. subuzbekistanica *Kond.*, Aru/04468 var. subvulgaris *Brsch.*, 7698/Bakanasski *var. subvulgaris Brsch.*, 7698/Bakanasski *var. italica Alef.*, Aru/7701 *var. vulgaris Koern.*, Bakanasski/0327 *var. vulgaris Koern.*, Aisaule/7679 *var. subvulgaris Brsch.*, 7698/Aisaule *var. italica Alef.*, 7698/Aisaule *var. suberythroseros Kan.*, Aru/7702 *var. erythroceros Koern.*, Aisaule/7689 *var. zeravschanica Brsch.*, Aru/0327 *var. vulgaris Koern.*, Aru/0327 *var. subpyrocarpa Alef.*, Bakanasski/7667 *var. subjanthoseros Brsch.*, Bakanasski/7667 *var. vulgaris Koern.*, Bakanasski/7701 *var. vulgaris Koern.*, Aisaule/7664 *var. italica Alef.*, Aisaule/7664 *var. subvulgaris Brsch.*, Aisaule/04470 *var. subvulgaris Brsch.*, Aisaule/04470 *var. italica Alef.*, Aisaule/Don 7712 *var. sundensis Koern.*, Aisaule/Don 7712 *var. italica Alef.*, Fatima/7695 *var. italica Alef.*, Fatima/7695 *var. breviaristata Vav.*, Bakanasski/7683 *var. subjanthoseros Brsch.*, 7698/Aru *var. erythroceros Koern.*, 7698/Aru *var. suberythroseros Kan.*, Bakanasski/Don 7712, Bakanasski/Don 7712 *var. Desvauxii Koern.*, Aru/212-05 *var. vulgaris Koern.*,

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Aru/7668 *var. vulgaris Koern.*, Aisaule/04468 *var. italica Alef.*, Aru/04636 *var. vulgaris Koern.*, Bakanasski/7690 *var. subjanthoseros Brsch.*, Bakanasski/7690 *var. vulgaris Koern.*, Bakanasski/04468 *var. subvulgaris Brsch.*, Bakanasski/04468 *var. vulgaris Koern.*, Aru/7666 *var. suberythroseros Kan.*, Fatima/7653 *var. persica Kan.*, Fatima/7689 *var. italica Alef.*, Aru/Don 7712 *var. erythroceros Koern.*, Aru/Don 7712 *var. Desvauxii Koern.*, Aru/Don 7712 *var. vulgaris Koern.*, Aru/Don 7712 *var. Desvauxii Koern.* semi-awned, ♀ Aru/7683 *var. janthoceros Koern.*, Aru/7683 *var. vulgaris Koern.*, Aisaule/7663 *var. italica Alef.*) obtained by crossing were used for analysis.

4.2. Hybridization Method

Hybridization was carried out using pneumocastration (three-channel pneumocastration) and the "TWEL" plant pollination method [54]. 31 genotypes were used as donors, the genome of which contains genes of resistance to rice blast (*Pi-1*, *Pi-2*, *Pi-33* and *Pi-ta*), and recipients were highly productive zoned local cultivars, such as Bakanasski, Fatima, Aisaule and Aru.

4.3. DNA Extraction and Multiplex PCR Analysis

Genomic DNA was extracted from two-week-old seedlings using the cetyltrimethylammonium bromide (CTAB) method [55]. CTAB reagent was obtained from Pan-Reac AppliChem GmbH (Darmstadt, Germany). All PCR analysis were performed using a T100 thermal cycler (BioRad, Germany). Multiplex PCR analysis was performed by adding several primers in PCR mix to determine the presence of two or more genes in a genotype. Each microtube contained 15 µl of PCR reaction mixture consisting of 40–50 ng of template DNA, 1.2 µl of each forward and reverse primer, 1.2 µl of *Taq* buffer, 0.05 mmol/L dNTPs and 1.0 U of *Taq* polymerase. The following PCR conditions were used: 94°C for 5 min; 35 cycles of 35 sec at 94°C, 45 sec at 60°C, and 30 sec at 72°C; and an extension of 5 min at 72°C. SSR (simple sequence repeat) markers were used to identify genes. The list of SSR markers (Syntol, Russia) for *Pi* complex genes is given in Table 3. The annealing temperature is the same for all primers, 60º C.

Table 3. List of tested SSR primers for *Pi* genes of the blast resistance complex.

Gene	Localization on chromosome	Name of a marker	Forward primer (5′-3′) Reverse sequence (5′-3′)
Pi-1	11	Rm 224	F - atcgatcgatcttcacgagg R - tgctataaaaggcattcggg
		Rm144	F - tgccctggcgcaaatttgatcc R - gctagaggagatcagatggtagtgcatg
Pi-2	6	Rm 527	F - ggctcgatctagaaaatccg R - ttgcacaggttgcgatagag
		SSR 140	F - aaggtgtgaaacaagctagcaa R - ttctaggggaggggtgtgaa
Pi-33	8	Rm 310	F - ccggcgataaaacaatgag R - gcatcggtcctaactaaggg
		Rm 72	F - ccggcgataaaacaatgag R - gcatcggtcctaactaaggg
Pi-ta	12	Pita	F1 - gccgtggcttctatctttacatg R1 - atccaagtgttagggccaacattc

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F2 - ttgacactctcaaaggactgggat

R2 - tcaagtcaggttgaagatgcatcga

The following genotypes were used as differentiator cultivars that are carriers of resistance genes: C101-Lac (+) for *Pi-1*, *Pi-33* genes, C101-A51 (+) for *Pi-2* gene and IR 36 (+) for *Pi-ta* gene. The cultivar Flagman (-) was used as a negative control for all studied genes.

4.5. Gel Electrophoresis

The amplification products were separated in 8% polyacrylamide gel (PAGE) in 1.0× TBE buffer by vertical electrophoresis chamber (Helicon, Russia). Electrophoretic gels were examined using the GelDoc XR (Bio-Rad, USA). The GelAnalyzer program was used to analyze gels.

4.6. Phytopathological Screening

Testing of 54 hybrid rice lines for resistance to *Magnaporthe oryzae* populations was carried out in 2023 in the artificial infectious nursery at the FSBSI "Federal Scientific Rice Centre" (Russia, Krasnodar). The most resistant cv. Avangard served as a positive control, and the highly susceptible cv. Pobeda 65 was used as a negative control.

Rice plants were infected with the fungal culture at the booting stage by spraying with a suspension of a mixture of conidia of 15 isolates of *M. oryzae*.

The degree of plant damage (in percentage) was considered on the 14th day after inoculation, according to the express method for assessing rice cultivars resistance to blast. The assessment was carried out considering the type of reaction (in points) on a ten-point scale (0-9) of the Standard Evaluation System for Rice [56]. The degree of resistance was indicated using the following designations: resistant lines - R, moderately resistant - MR and susceptible - S.

The intensity of disease development (IDD, %) was calculated using the formula:

IDD = $\sum$ (a*b)/ n*9,

where - $\sum$ (a*b) – sum products of the number of infected plants multiplied by the corresponding damage score, n - the number of recorded plants, pieces.

4.7. Analysis of Structural Elements of Yield of Hybrid Rice Lines

Evaluation of the productivity of hybrid lines in comparison with the standard cv. Bakanasski was carried out according to the biometric parameters of the elements of yield structure ("bushiness, pc", "plant height, cm", "panicle length, cm", "number of grains from the main panicle, pc", "weight of seeds from the main panicle, g", "weight of 1000 seeds, g" and etc.) [57]

4.8. Statistical Analyses

To characterize the productivity of hybrid lines in comparison with the cv. Bakanasski, which is the standard for the Almaty region, statistical indicators such as the mean value, standard deviation for the main traits characterizing rice productivity, "weight of 1000 seeds, g" were used. Analysis of variance (one-way ANOVA) was performed to compare the mean values of the data groups. Data processing was carried out in Microsoft Excel 2021 using a data analysis package.

5. Conclusions

Breeding programs successfully use both traditional breeding methods and modern MAS analysis tools involving a wide range of genetic diversity. In the context of global climate change, the creation of elite rice lines and varieties resistant to adverse environmental factors is a necessity to meet the ever-increasing demand for food products. It is known that over time, most varieties that have one resistance gene in their genotype cannot withstand changing fungal races and lose resistance, which leads to a decrease in rice yield. In connection with the above-described problem, increasing rice resistance by pyramiding several resistance genes in one genotype is crucial for stable

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rice production. The created pyramidal lines are of great value in rice breeding for improving the resistance of local varieties.

The use of modern MAS technologies guarantees the acceleration of rice breeding and hence the introduction to the market of competitive rice cultivars with durable immunity to disease and other stress factors. Microsatellite markers are more often used to assess polymorphism and genetic diversity of samples [58]. The created new pyramided lines of rice that are resistant to phytopathogens will be used in rice breeding to create cultivars, which increases the yield and competitiveness of rice in the market and promotes the export of rice products. These pyramidal lines will be used as a source of resistance genes in the breeding process and for germplasm exchange.

Rice is the food source for most people on the planet. The world's population is increasing every year. Improving rice resistance to blast by introgression and pyramiding of genes will help maintain rice yields during epiphytotic periods, which contributes to sustainable agricultural development and achieving zero hunger according to the UN program [https://www.un.org/sustainabledevelopment/sustainable-development-goals/].

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