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ARTICLE

Conventional Breeding and Molecular Markers for Blast Disease Resistance in Rice (*Oryza sativa* L.)

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ABSTRACT

Monogenic lines, which carried 23 genes for blast resistance were tested and used donors to transfer resistance genes by crossing method. The results under blast nursery revealed that 9 genes from 23 genes were susceptible to highly susceptible under the three locations (Sakha, Gemmeza, and Zarzoura in Egypt); *Pia, Pik, Pik-p, Piz-t, Pita, Pi b, Pi, Pi 19* and *Pi 20*. While, the genes *Pii, Pik-s, Pik-h, Pi z, Piz-5, Pi sh, Pi 3, Pi 1, Pi 5, Pi 7, Pi 9, Pi 12, Pikm* and *Pita-2* were highly resistant at the same locations. Clustering analysis confirmed the results, which divided into two groups; the first one included all the susceptible genes, while the second one included the resistance genes. In the greenhouse test, the reaction pattern of five races produced 100% resistance under artificial inoculation with eight genes showing complete resistance to all isolates. The completely resistant genes: *Pii, Pik-s, Piz, Piz-5* (=*bi2*) (t), *Pita* (=*Pi4*) (t), *Pita*, *Pi b* and *Pi1* as well as clustering analysis confirmed the results. In the F_1 crosses, the results showed all the 25 crosses were resistant for leaf blast disease under field conditions. While, the results in F_2 population showed seven crosses with segregation ratio of 15 (R):1 (S), two cross gave segregated ratio of 3 R:1 S and one gave 13:3. For the identification of blast resistance genes in the parental lines, the marker K3959, linked to *Pik-s* gene and the variety IRBLKS-F5 carry this gene, which was from the monogenic line. The results showed that four genotypes; Sakha 105, Sakha 103, Sakha 106 and IRBLKS-F5 were carrying *Pik-s* gene, while was absent in the Sakha 101, Sakha 104, IRBL5-M, IRBL9-W, IRBLTACP1 and IRBL9-W(R) genotypes. As for *Pi 5* gene, the results showed that it was present in Sakha 103 and Sakha 104 varieties and absent in the rest of the genotypes. In addition, *Pita-Pita-2* gene was found in the three Egyptian genotypes (Sakha 105, Sakha 101 and Sakha 104) plus IRBLTACP1 monogenetic. In F_2 generation, six populations were used to study the inheritance of blast resistance and specific primers to confirm the ratio and identify the resistance genes. However, the ratios in molecular markers were the same of the ratio under field evaluation in the most population studies. These findings would facilitate in breeding programs for gene pyramiding and gene accumulation to produce durable resistance for blast using those genotypes.

KEYWORDS

Biotechnology tools; clustering analysis; monogenic lines; resistance genes; breeding; *Oryza sativa* L.

1 Introduction

Many pathogens attack the rice crop, causing diseases with different symptoms that vary from species to species [1,2]. However, the most devastating one is blast disease, caused by *Magnaporthe oryzae*, which infects the leaves and panicles results in a huge loss in yield [3]. The estimation of the yield losses was recorded from 5% to 70%, according to the severity of infection [4–6]. Crop loss through blast disease has been estimated worldwide as sufficient as food for approximately 60 million people, and this crop loss was also estimated to be approximately $70 billion.

The blast disease infection is affected by different factors such as the number of resistance genes in the host cultivar, cultural practices, and climate change [7]. Resistance genes are used as a genetic tool to control the inheritance of blast disease characteristics and are considered as a cost-effective and environmentally beneficial mean for minimizing crop losses caused by disease. So far, more than 100 major blast resistance genes and 300 quantitative trait loci (QTL) have been identified and documented [8,9]. Many rice varieties have complete resistance to the blast, however, after a few cultivated years; the resistance has been broken down due to blast race changes and the appearance of newer, more virulent isolates of rice blast fungus [10]. Conventional breeding, thus, is still the mainstay for managing blast resistant cultivars to develop highly resistant cultivars to manage the disease. In addition it is a good method to understand the relationships between races and the resistance genes studied [11,12]. As a result, one of the goals of the breeding programs is to use resistance varieties as donors to transfer resistance genes to offspring, which can also be used to obtain durable resistance through gene pyramiding. The developed Japanese differential varieties (JDV) were the first varieties used as donors for blast resistance genes and have also been used to identify the individual genes responsible for blast resistance [13]. Development of monogenic lines with a single blast resistance genes were produced through backcross breeding method. Varieties and lines with known gene(s) for resistance were used as donors. Lijianxintuanheigu (LTH) was used as a recurrent parent in the backcrossing, through this method 23 resistance genes were transfer as individual Lijianxintuanheigu variety [14]. Therefore, the identification of blast resistance genes in the cross-parents is very important to transfer the resistance trait and accumulating many different resistance genes in a new variety, which can produce durable resistance. The molecular marker technology was used to identify and confirm the blast resistance genes and detect the chromosomal regions associated with such characters [15,16]. Many different molecular markers have been used, such as microsatellite or simple sequence repeat (SSR), inter-simple sequence repeat (ISSR), intron-exon splice junction (ISJ), Single Nucleotide Polymorphism (SNP), Sequence Tagged Microsatellites (STMS) and Sequence Tagged Sites (STS) to identify blast resistance genes [17–19]. The objectives of this study were to: 1) Use hybridization to transfer the blast resistance genes from monogenic lines to some Egyptian rice varieties; 2) Investigate the transmission of blast traits through the second generation; and 3) Use SNP and SSR markers to identify blast resistance genes in the parents under study and confirm the ration in the F_2 generation.

2 Materials and Methods

This study was conducted at the experimental farm of the Rice Research and Training Center (RRTC), Sakha, Kafr El-Sheikh, Egypt and Agricultural Biotechnology Department, King Faisal University during the years 2018 to 2021. Ten parents were used as the genetic materials in this investigation (Table 1), including five Egyptian commercial varieties as parental lines, and five monogenic lines (carry different resistance genes) as "testers."

2.1 Field Evaluation

The blast resistance evaluations for the thirty monogenic lines were achieved during the 2018 season with both natural infections at blast nursery test in three different locations (Sakha, Gemmeza, and Zarzoura) and artificial inoculation in the greenhouse and five of them were selected as testers (Table 1).

Table 1: Rice genotypes, parentage, origin, blast reaction and resistance genes

No.	Genotypes	Parentage	Origin	Blast reaction	Resistance gene
1	Sakha 105	Gz5581/Gz4316	Egyptian	R	-
2	Sakha 101	(Giza 176/Milyang79)	Egyptian	S	-
3	Sakha 103	(Sakha105/SUWEON 349)	Egyptian	R	-
4	Sakha 104	(GZ 4096/GZ 4100)	Egyptian	S	-
5	Sakha 106	Sakha105/Hexi30	Egyptian	R	-
6	IRBLKS-F5	Lijiangxintuanheigu/FUJISAKA 5	IRRI	R	*Pik-s*
7	IRBL5-M	Lijiangxintuanheigu/RIL 249	IRRI	R	*Pi 5(t)*
8	IRBL9-W	Lijiangxintuanheigu/WHD-1S-75-1-127	IRRI	R	*Pi 9(t)*
9	IRBLTACP1	Lijiangxintuanheigu/C101PKT	IRRI	R	*Pita*
10	IRBLZ5-CA(R)	Lijiangxintuanheigu/AICHI ASAHI	IRRI	R	*Piz-5*

2.2 Greenhouse Evaluation under Artificial Inoculation

Seeds of monogenic lines varieties were seeded in plastic trays (30 cm × 20 cm × 15 cm). Each tray included 20 rows representing fifteen monogenic lines and five local varieties. The rest of the monogenic lines were sown in other trays. The trays were kept in the greenhouse and fertilized with Urea 46.5% N (5 g/tray). Seedlings were inoculated after 29 days from the sowing date. The seedlings were artificially inoculated with five rice blast isolates, i.e., Eg-5, IG-1, IB-45, 367, and 374. All tested lines were sprayed with spore suspension (100 mL) adjusted to 5×10^4 spores/mL. Each isolate was sprayed using an electrical spray gun. The inoculated seedlings were held in a moist chamber with more than 90% R.H. and 25°C–28°C for 24 h, and then moved to the greenhouse. Seven days after inoculation, blast reactions were recorded according to the standard evaluation system using a 0–9 scale [20].

2.3 Field Experiments

Ten parental varieties (5 Egyptian as lines and selected 5 monogenics as testers) in this study were sown at three sowing dates at fifteen-day intervals to overcome the difference in heading dates among the parental varieties. At 30 days of age, the seedlings of the parents were transplanted to the experimental field in 3 rows, each five meters long and 20 cm apart between plants in each row. A line × tester cross was conducted among the 10 parents to produce 25 crosses. The hybridization technique was done according to Jodon [21] and Butany [22]. All the materials were arranged in a randomized complete block design (RCBD) with three replications; each replication contained 25 individual plants. The 25 populations of F_2 materials, each consisting of more than 240 individual plants, were planted and evaluated for blast nursery.

2.4 Blast Scoring at Blast Nursery Test

In the blast nursery, all the breeding materials (parents, F_1 and F_2) were tested against the natural infestation of blast races under the optimum conditions for blast infection, like the delay of sowing dates, increasing the amount of nitrogen fertilization, growing susceptible check (blast spreader), growing and applying in different locations. The plants' materials were evaluated for their reaction against *M. grisea* at the blast nursery in three locations: Sakha, Gemmiza, and Zarzora during 2019, 2020, and 2021 seasons for blast resistance at the seedling stage. About forty days from the sowing date, the typical blast lesions

were scored according to the Standard Evaluation System using a 0–9 scale [20] as follows: 1–2 = resistant (R), 3 = moderately resistant (MR), 4–6 = susceptible (S), and 7–9 = highly susceptible (HS).

The quantities and qualitative characters; leaf blast reaction at seedling stage (LBR), heading date (DH-day), plant height (PH-cm), number of panicles/plant (NPP), Spikelet fertility% (SF), 1000-grain weight (1000GW-g), grain yield/plant (GY-g), panicle weight (PW-g) and number of primary branches were evaluated for F1 according to the Standard Evaluation System IRRI [20]. As well as 25 F_2 populations at an adult stage were evaluated to study the inheritance of blast resistance traits in the segregating populations. The Chi-square x^2 test was computed as follows [23]. The *t*-test was used to examine the existence of genetic variance between parental means.

Correlation of traits: Correlation among the values of the traits estimated was calculated using Pearson correlation coefficients and plotted via the packages corrplot and Performance Analytics [24].

2.5 Molecular Marker Analysis

Ten parental genotypes and six F_2 populations were employed in the study to identify the blast resistance genes using seven specific primers purchased from Sangon Company, China. DNA Genomic extraction of the parental genotypes and F_2 were carried out [25]. Seven markers (Table 2) were used for this investigation. Six of them were Single Nucleotide Polymorphism (SNP) and one was an SSR marker.

Table 2: Specific markers and resistance genes for blast

No.	Type of marker	Marker name	R. genes	Chromosome No.	Sequence*
1	**SNP**	**k3957**	*Pik-P*	1	F: CTCAAGATTGTATCGTCGACGACTA R: GAGAGGTTTGCAGCCAGACCAGG
2	**SNP**	**JJ817**	*Pi 5(t)*	9	F: GATATGGTTGAAAAGCTAATCTCA R: ATCATTGTCCTTCATATTCAGAGT
3	**SNP**	YL153/YL154	*Pita,* *Pita*-2	12	F: CAACAATTTAATCATACACG R: ATGACACCCTGCGATGCAA
4	**SNP**	**JJ81-T3**	*Pi 3(t)*	9	F: TCTACAAACTCAGTTAAACT R: AGCGAAAATCATTTATCACA
5	**SNP**	**JJ113-T3**	*Pii (t)*	9	F: CTCTTGGTGATCTTTGTTAC R: GGATGATGTGATCTGCAGAG
6	SSR	**RM3843**	*Pi 39*	4	F: ACCCTACTCCCAACAGTCCC R: GGGGTCGTACGCTCATGTC
7	SNP	**T8042**	*Pit*	1	F: CTCAAGATTGTATCGTCGACGACTA R: GAGAGGTTTGCAGCCAGACCAGG

Note: * F = forwarded primer, R = reverse primer.

2.6 Polymerase Chain Reaction (PCR) Assay

The reaction mixture (25 µL) consisted of: 12.5 µL of 2× master mix ready to use {0.1 U/µL Taq Polymerase, 20 mM Tris-HCl (pH8.3), 100 mM KCl, 3 mM $MgCl_2$, and stabilizer and enhancer)}, 10 Pmol of each primer (1.0 µL), 1.0 uL of DNA (50 ng) and 9.5 µL of PCR grade water. Amplifications were performed in a thermocycler (Bio-Rad, C-1000, California, USA) as follows: (1) initial denaturation at 94°C for 5 min; (2) 30 s of denaturation at 94°C; (3) primers' annealing temperatures differ according to their Tm, for 1 min; (4) one-minute extension at 72°C; (5) Steps 2, 3, and 4 are repeated 40 times; (6) a final extension of 10 min at 72°C was given. The amplified products were analyzed on a 2% agarose

gel electrophoresis containing ethidium bromide and then photographed and analyzed using BioDoc Analysis software (Biometra, Germany).

3 Results and Discussion

3.1 Scoring of Monogenic Lines under Natural Infection

The blast reactions of monogenic lines were tested at three locations in the blast nursery and the results are shown in Table 3. The tested monogenic lines exhibited different reactions to the dominant races in the blast nurseries and natural fields according to the response of their genes (Table 3). However, the reaction revealed that 10 out of 23 genes were susceptible to highly susceptible conditions at three locations, while the rest of the genes were highly resistant at the same locations (Table 3).

Table 3: Reaction of monogenic lines under blast nursery test in Sakha, Gemmiza and Zarzoura locations

S. No.	Monogenic lines	Locations			Blast reaction	Resistance gene
		Sakha	Gemmiza	Zarzoura		
1	IRBLA-A	6	7	6	S	*Pi a*
2	IRBLA-C	5	6	5	S	*Pi a*
3	IRBLI-F5	1	1	1	R	*Pi i*
4	IRBLKS-F5	1	1	1	R	*Pik-s*
5	IRBLKS-S	1	1	1	R	*Pik-s*
6	IRBLKKA	5	5	5	S	*Pik*
7	IRBLKP-K60	4	4	4	S	*Pik-p*
8	IRBLKH-K3	1	1	1	R	*Pik-h*
9	IRBLZFU	1	1	1	R	*Pi z*
10	IRBLZ5-CA	1	1	1	R	*Pi z-5(=bi2)(t)*
11	IRBLZT-T	5	5	5	S	*Pi z-t*
12	IRBLTA-K1	4	4	4	S	*Pi ta(=pi4)(t))*
13	IRBLTACT2	1	1	1	R	*Pita*
14	IRBLB-B	4	4	4	S	*Pi b*
15	IRBLT-K59	4	4	4	S	*Pi*
16	IRBLSH-S	1	1	1	R	*Pi sh*
17	IRBLSH-B	1	1	1	R	*Pi sh*
18	IRBL1-CL	2	2	3	R	*Pi 1*
19	IRBL3-CP4	1	1	1	R	*Pi 3*
20	IRBL5-M	1	1	1	R	*(Pi 5(t))*
21	IRBL7-M	1	1	1	R	*(Pi 7(t))*
22	IRBL9-W	1	1	1	R	*(Pi 9(t))*
23	IRBL12-M	2–3	2	2	R	*(Pi 12(t))*
24	IRBL19-A	5	5	5	S	*(Pi 19(t))*
25	IRBLKMTS	1	1	1	R	*Pik-m*

(Continued)

Table 3 (continued)

S. No.	Monogenic lines	Locations			Blast reaction	Resistance gene
		Sakha	Gemmiza	Zarzoura		
26	IRBL20-IR24	4	4	4	S	*Pi 20*
27	IRBLTA2-PI	1	1	1	R	*Pita-2*
28	IRBLTACP1	1	1	1	R	*Pita*
29	IRBL11-ZH	1	1	1	R	*(Pi 11(t))*
30	IRBLZ5-(CA(R))	1	1	1	R	*Piz-5*

Note: 1–2 = resistant (R), 3 = moderately resistant (MR), 4–6 = susceptible (S) and 7–9 = highly susceptible (HS).

Some alleles of the *Pik* gene were resistant, while others are susceptible at the same location. The results demonstrated that the most effective genes under both natural and artificial inoculation were *Piz* and *Pii*. These two genes are resistant under Egyptian conditions in the three locations. Therefore, they can be employed to solve the broken-down resistance varieties. Clustering analysis divided all the genotypes into two groups (Fig. 1). The first group included all the susceptible genes, while the second group included the resistance genes. Generally, resistance genes for blast commonly called *Pi* were helping and providing a broad-spectrum resistance against the most prevalent races and can be extremely valuable in rice breeding efforts [6,26].

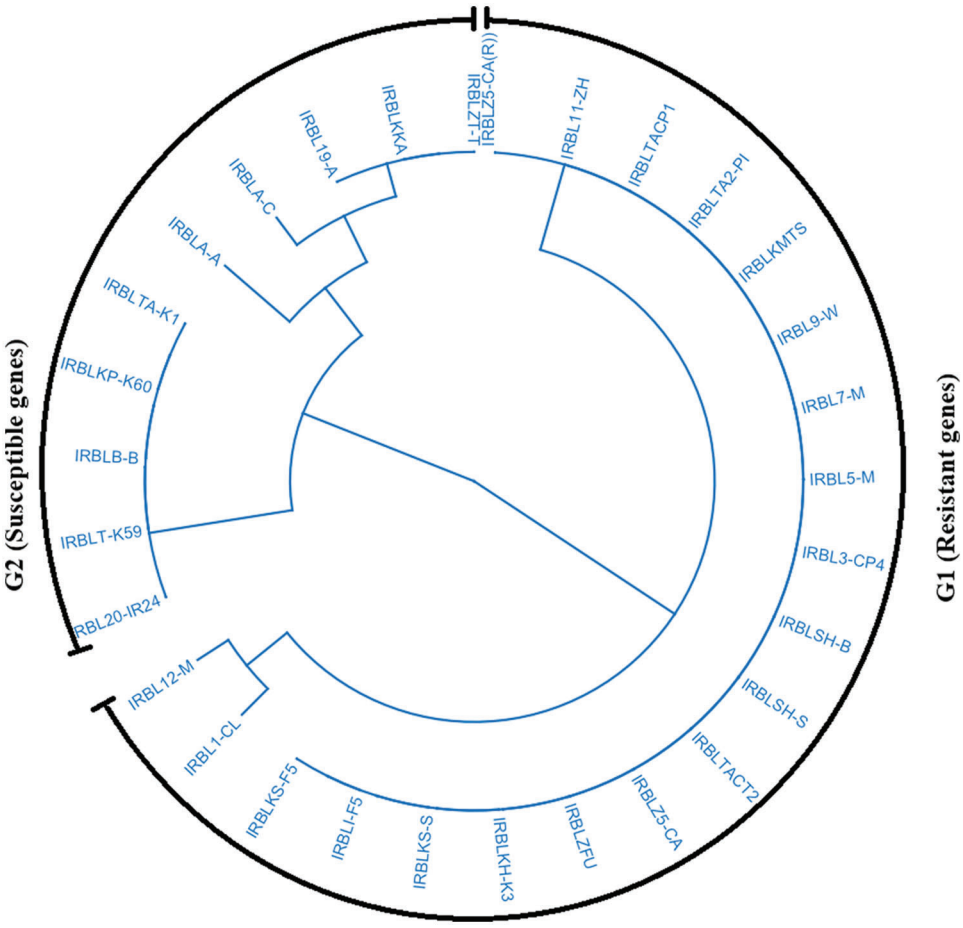

Figure 1: Dendrogram for monogenic lines according to the reactions in different locations

In addition, it has become necessary to know and identify these genes, to be used in hybridization to transfer the resistance trait into new varieties [3,11]. In another context, the modern or new varieties are better to carry multiple resistance genes to display broad-spectrum and durable resistance in the field or gene accumulation and gene pyramiding. In any case, the traditional method was used for producing monogenetic lines to make crosses between them and the commercial varieties whose resistance is required to be improved, and with molecular markers to identify resistance genes.

3.2 Evaluation of Monogenic Lines under Artificial Infection

Five specific blast races were used to evaluate monogenic lines under greenhouse conditions. The selected five isolates were classified as pathogenic and were used as differential blast isolates to estimate resistance genes by reaction pattern. The isolate Eg-5 was used as a highly virulent and most aggressive isolate for different local varieties (Table 4).

Table 4: Reaction of monogenic lines under artificial inoculation in greenhouse

S. No.	Monogenic lines	Blast races					Resistant (%)	Resistance gene
		Eg-5	IG-1	367	374	IB-45		
1	IRBLA-A	4	2	4	2	2	60%	*Pia*
2	IRBLA-C	2	2	4	2	2	80%	*Pia*
3	IRBLI-F5	2	2	2	2	2	100%	*Pii*
4	IRBLKS-F5	3	2	4	2	2	80%	*Pik-s*
5	IRBLKS-S	4	2	2	2	2	80%	*Pik-s*
6	IRBLKKA	4	2	2	2	2	80%	*Pik*
7	IRBLKP-K60	6	2	2	2	2	80%	*Pik-p*
8	IRBLKH-K3	4	2	2	2	2	80%	*Pik-h*
9	IRBLZFU	2	2	2	2	2	100%	*Piz*
10	IRBLZ5-CA	1	2	2	2	2	100%	*Piz-5 = (bi2)(t)*
11	IRBLZT-T	4	2	2	2	4	60%	*Piz-t*
12	IRBLTA-K1	1	2	2	2	2	100%	*Pita = (pi4)(t)*
13	IRBLTACT2	2	2	2	2	2	100%	*Pita*
14	IRBLB-B	2	2	2	2	2	100%	*Pi b*
15	IRBLT-K59	4	4	2	2	2	60%	*Pi*
16	IRBLSH-S	5	2	4	2	2	60%	*Pi sh*
17	IRBLSH-B	4	2	4	2	2	60%	*Pi sh*
18	IRBL1-CL	2	2	2	3	2	100%	*Pi 1*
19	IRBL3-CP4	5	2	2	2	2	80%	*Pi 3*
20	IRBL5-M	4	2	2	2	2	80%	*Pi 5(t)*
21	IRBL7-M	6	2	2	2	4	60%	*Pi 7(t)*
22	IRBL9-W	2	2	3	2	4	80%	*Pi 9(t)*
23	IRBL12-M	2	2	4	2	2	80%	*Pi 12(t)*
24	IRBL19-A	7	2	2	2	5	60%	*Pi 19(t)*

(Continued)

Table 4 (continued)

S. No.	Monogenic lines	Blast races					Resistant (%)	Resistance gene
		Eg-5	IG-1	367	374	IB-45		
25	IRBLKMTS	2	2	5	2	2	80%	*Pik-m*
26	IRBL20-IR24	5	2	3	2	2	80%	*Pi 20*
27	IRBLTA2-PI	2	2	4	2	2	80%	*Pita-2*
28	IRBLTACP1	2	2	2	2	2	80%	*Pita*
29	IRBL11-ZH	2	2	2	2	2	80%	*Pi 11(t)*
30	IRBLZ5-CA(R)	4–5	2	5	2	2	60%	*Piz-5*

The results showed that, according to the reaction patterns of five races under artificial inoculation, seven genes (*Pii, Piz, Piz-5 = (bi2)(t), Pita = (Pi4)(t), Pita, Pi b,* and *Pi 1*) showed complete resistance to all isolates. Only five of the seven completely resistant genes (*Pii, Pik-s, Piz, Piz-5 = (bi2)(t)*, and *Pita = (Pi4)(t)*) were resistant and effective under both natural and artificial blast nursery conditions. These results indicated that the monogenic lines that carry these genes could be recommended to be used as donors in breeding programs to improve resistance to blast disease. On the other hand, the genes *Pia, Pik-s, Pik, Pik-p, Pik-h, Pi 3, Pi 5(t), Pi 9(t), Pi 12(t), Pik-m, Pita-2, Pita* and *Pi 11(t)* gave 80% resistance, while the genes *Pia, Piz-t, Pi, Pi sh, Pi 7(t), Pi 19(t)* and *Piz-5* gave 60% resistance (Table 4). These results were found with [27–30].

Clustering analysis (Fig. 2) confirmed the results, which were divided into two major groups. The first one included IRBL7-M and IRBL19-A, which carry the *Pi 7* and *Pi 19* genes, and the resistance percentage was 60% under the five races in the greenhouse test, while, the second group was divided into two sub-groups. The first sub-group included *Pia, Pik-s, Pik-p*, and *Pi 5* genes, and the second sub-group included *Pia, Pita-2, Pik-m, Pii, Piz, Pi b, Piz-5,* and *Pi (t)*.

The rice breeder can use the parents between clusters as parents for future hybridization rather than within clusters for a successful breeding program and selection of genetically diverse parents as an important pre-requisite to obtain better and desirable recombinants. These results agreed well with earlier researchers [31–33].

3.3 ANOVA Analysis

The result of ANOVA analysis (Table 5) was performed to test the difference between the parents and hybrids for all the studied traits. Results revealed that mean squares due to genotypes were significant for all the traits. The mean squares due to genotypes were further partitioned into parents, crosses and parents *vs.* crosses. The differences among parents were highly significant for all traits, indicating the presence of wide genetic variability among parents for almost all the traits. The mean square values due to crosses for all traits were found to be significant at 0.01 levels. Parents *vs.* crosses mean square values further revealed highly significant differences in all crosses. In addition, male testers exhibited highly significant differences for all traits. The highly significant mean squares of lines × testers for all traits indicated that they interacted and produced markedly different combining ability effects, and this might be due to the wide genetic diversity of lines and testers. In addition, the mean squares due to lines *vs.* testers were significant for all traits, which indicated that female and male parents differed significantly for these traits. These results are similar to those obtained by [34,35].

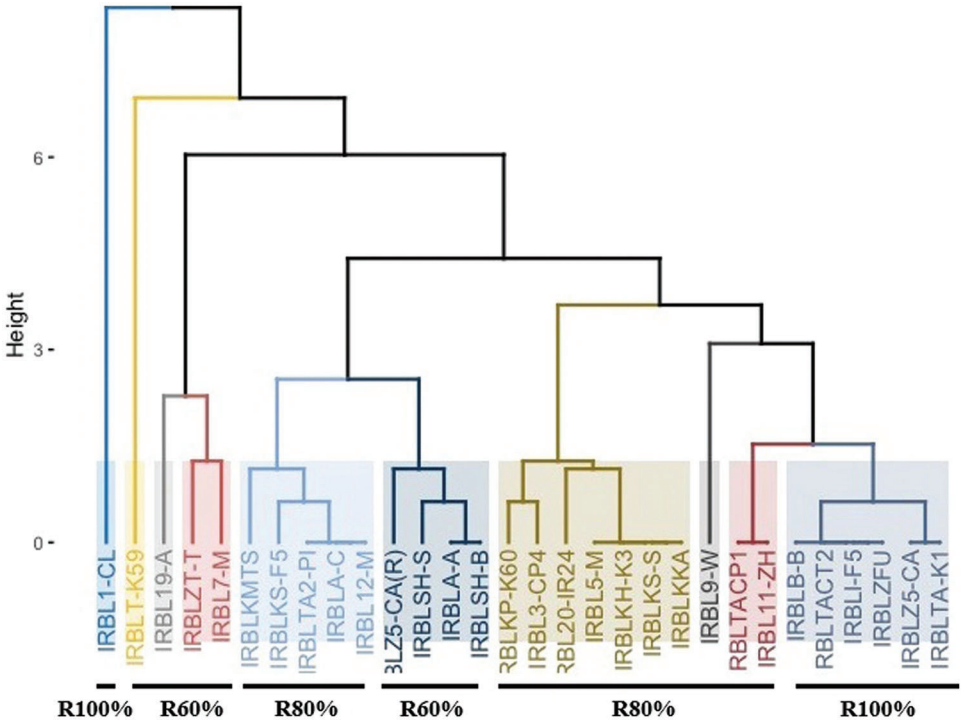

Figure 2: Cluster analysis for monogenic lines according to the artificial inoculation in greenhouse

Table 5: Analysis of variance and mean square from line × testers analysis for the studied traits

S.O.V	D.F	Duration	Plant height (cm)	Flag leaf area (cm^2)	No. of panicles $plant^{-1}$
Reps.	2	2.600	1.371	0.200	0.800
Genotypes	34	298.649**	1250.259**	116.430**	441.827**
Parents	9	183.86**	670.700**	122.03**	21.317**
P.Vs.C	1	652.937**	13759.62**	818.42**	7318.086**
Crosses	24	326.930**	946.370**	85.080**	313.008**
Lines	4	1202.580**	993.720**	134.580**	879.813**
Testers	4	384.780**	3582.420**	181.380**	183.513**
Line × testers	16	93.555**	275.520**	48.630**	203.680**
Error	68	0.953	0.989	1.024	1.192
L.S.D. 5%		1.59	1.62	1.65	1.78
1%		2.12	2.15	2.19	2.37

(Continued)

Table 5 (continued)

Contu.

S.O.V	D.F	Panicle weight (gm)	1000-grain weight (gm)	Spikelet fertility (%)	Grain yield plant^{-1}
Reps.	2	0.004	0.059	1.165	20.492
Genotypes	34	3.901**	17.63**	255.271**	964.665**
Parents	9	4.201**	32.75**	129.334**	604.295**
P.Vs.C	1	35.587**	4.69**	190.848**	1742.821**
Crosses	24	2.469**	12.499**	305.181**	1067.380**
Lines	4	0.680**	21.954**	418.587**	3885.247**
Testers	4	6.013**	16.297**	596.953**	633.780**
Line × testers	16	2.030**	9.185**	203.887**	471.313**
Error	68	0.008	0.013	0.752	3.331
L.S.D. 5%		0.14	0.18	1.41	2.98
1%		0.19	0.24	1.88	3.96

Note: *, ** Significant at 0.05 and 0.01 levels, respectively.

3.4 Mean Performance

The results in Table 6 showed the mean performance of parents, lines, and their hybrids for the studied blast resistance traits. All testers showed high resistance to leaf blast and carried different resistance genes (*Pik-s, Pi 5 (t), Pi 9 (t), Pita,* and *Piz-5*, respectively). Furthermore, all varieties were resistant, except the Sakha 101 and Sakha 104 varieties, which were highly susceptible to the leaf blast trait, while F_1 crosses recorded resistance to leaf blast disease under field conditions. Moreover, the results revealed that all crosses that were driven by parents' resistance × resistance parents and resistance × susceptible parents were all resistant in F_1. These results indicated that the parents carried dominant genes for resistance and that resistance was completely dominant over susceptibility to blast. For agronomic traits, the mean performance of parents, lines, and their hybrids for studied traits are presented in Table 6.

Table 6: Mean performance of parents and their F_1 hybrid for studied traits

Genotypes (S. No.)	Leaf blast reaction	Duration (days)	Plant height (cm)	Flag leaf area (cm^2)	No. of panicles plant^{-1}
Lines					
Sakha 105 (1)	R	123	97	28	17
Sakha 101 (2)	S	142	92	34	23
Sakha 103 (3)	R	122	98	32	19
Sakha 104 (4)	S	134	105	32	21
Sakha 106 (5)	R	133	99	38	24

(Continued)

Table 6 (continued)

Genotypes (S. No.)	Leaf blast reaction	Duration (days)	Plant height (cm)	Flag leaf area (cm^2)	No. of panicles $plant^{-1}$
Testers					
IRBLKS-F5 (6)	R	117	98	32	20
IRBL5-M (7)	R	122	123	45	17
IRBL9-W (8)	R	121	140	27	18
IRBL9-W (9)	R	130	115	23	16
IRBLTACP1 (10)	R	118	100	26	21
F1 Hybrids					
Sakha 105 × IRBLKS-F5 (11)	R	127	102	36	37
Sakha 105 × IRBL5-M (12)	R	120	120	44	35
Sakha 105 × IRBL9-W (13)	R	118	153	30	34
Sakha 105 × IRBLTACP1 (14)	R	128	133	38	39
Sakha 105 × IRBL9-W(R) (15)	R	117	122	36	37
Sakha 101 × IRBLKS-F5 (16)	R	131	121	48	37
Sakha 101 × IRBL5-M (17)	R	133	145	43	35
Sakha 101 × IRBL9-W (18)	R	142	160	29	35
Sakha 101 × IRBLTACP1 (19)	R	145	122	36	34
Sakha 101 × IRBL9-W(R) (20)	R	147	140	40	36
Sakha 103 × IRBLKS-F5 (21)	R	116	105	35	26
Sakha 103 × IRBL5-M (22)	R	130	128	38	29
Sakha 103 × IRBL9-W (23)	R	117	150	33	32
Sakha 103 × IRBLTACP1 (24)	R	138	122	35	30
Sakha 103 × IRBL9-W(R) (25)	R	118	109	30	21
Sakha 104 × IRBLKS-F5 (26)	R	130	115	38	25
Sakha 104 × IRBL5-M (27)	R	131	118	37	36
Sakha 104 × IRBL9-W (28)	R	133	143	32	29
Sakha 104 × IRBLTACP1 (29)	R	145	144	37	34
Sakha 104 × IRBL9-W(R) (30)	R	135	136	41	38
Sakha 106 × IRBLKS-F5 (31)	R	132	113	45	36
Sakha 106 × IRBL5-M (32)	R	135	134	49	30
Sakha 106 × IRBL9-W (33)	R	140	165	43	28
Sakha 106 × IRBLTACP1 (34)	R	143	159	35	30
Sakha 106 × IRBL9-W(R) (35)	R	144	142	39	30

(Continued)

Table 6 (continued)

Genotypes (S. No.)	Leaf blast reaction	Duration (days)	Plant height (cm)	Flag leaf area (cm^2)	No. of panicles $plant^{-1}$
L.S.D. at 5%		1.54	1.42	1.59	1.74
at 1%		2.11	2.12	2.21	2.32

Cantu.

Genotypes (S. No.)	Panicle weight (gm)	1000-grain weight (gm)	Spikelet fertility (%)	Grain yield $plant^{-1}$ (gm)
Lines				
Sakha 105 (1)	3.5	28.2	93.2	44.6
Sakha 101 (2)	4.0	29.4	92	46.5
Sakha 103 (3)	3.1	24.3	92.4	43.3
Sakha 104 (4)	4.1	28.4	95	42.3
Sakha 106 (5)	4.0	27.2	86	46.7
Tasters				
IRBLKS-F5 (6)	1.9	24.2	91.6	32.3
IRBL5-M (7)	1.6	26.3	73.1	25.2
IRBL9-W (8)	2.7	22.3	87.5	39.7
IRBL9-W (9)	2.8	18.3	82	13.5
IRBLTACP1 (10)	2.1	25.3	92.2	37
F1 Hybrids				
Sakha 105 × IRBLKS-F5 (11)	4.1	26.4	94.5	56.03
Sakha 105 × IRBL5-M (12)	4.3	28.5	95.5	54.6
Sakha 105 × IRBL9-W (13)	4.5	29.6	84.5	62.1
Sakha 105 × IRBLTACP1 (14)	3.9	26	81.4	41
Sakha 105 × IRBL9-W(R) (15)	3.7	29.2	90.9	59.3
Sakha 101 × IRBLKS-F5 (16)	4.1	26.1	91.7	47.7
Sakha 101 × IRBL5-M (17)	3.8	22.6	85.1	61.5
Sakha 101 × IRBL9-W (18)	4.2	25.7	80.8	51.3
Sakha 101 × IRBLTACP1 (19)	3.6	23	83.4	50.02
Sakha 101 × IRBL9-W(R) (20)	3.4	26.5	85.2	58.3
Sakha 103 × IRBLKS-F5 (21)	4.1	24.2	95	43.4
Sakha 103 × IRBL5-M (22)	4.1	26.4	92	32.4
Sakha 103 × IRBL9-W (23)	3.6	24.1	90	38.7
Sakha 103 × IRBLTACP1 (24)	3.6	27.6	94.5	38.5
Sakha 103 × IRBL9-W(R) (25)	4.4	26.7	92.1	37.7
Sakha 104 × IRBLKS-F5 (26)	3.3	28	81.5	37.8

(Continued)

Table 6 (continued)

Cantu.

Genotypes (S. No.)	Panicle weight (gm)	1000-grain weight (gm)	Spikelet fertility (%)	Grain yield plant^{-1} (gm)
Sakha 104 × IRBL5-M (27)	4.3	27	97.5	31.3
Sakha 104 × IRBL9-W (28)	3.7	24.5	71.5	37.5
Sakha 104 × IRBLTACP1 (29)	3.3	22	76	33.5
Sakha 104 × IRBL9-W(R) (30)	3.8	28.1	75.6	47.9
Sakha 106 × IRBLKS-F5 (31)	4.1	27	95.6	33.7
Sakha 106 × IRBL5-M (32)	4.02	25.3	92.5	40.7
Sakha 106 × IRBL9-W (33)	4.2	23.5	70.5	52.1
Sakha 106 × IRBLTACP1 (34)	2.9	24.2	91.9	30.4
Sakha 106 × IRBL9-W(R) (35)	4.04	26.4	55.7	38.05
L.S.D. at 5%	0.146	0.186	1.41	2.98
at 1%	01.94	0.247	1.88	3.96

Testers showed a considerable number of early values, and lines showed a lower number of values than testers. The genotypes IRBLKS-F5 and IRBLTACP1 had the shortest duration among the tester lines, while the Sakha 103 and Sakha 105 varieties were the earliest maturing varieties among the lines. The best combinations were Sakha 103 × IRBLKS-F5 (116 days), Sakha 105 × IRBL9-W (R) (117), Sakha 103 × IRBL9-W (117 days) and Sakha 103 × IRBL9-W (117 days). Regarding the yield traits for testers, lines, and hybrids (Table 6), in general, lines demonstrated higher values for panicle/plant, spikelet fertility%, 1000-grain weight, and grain yield/plant than testers. However, some hybrids indicated high values for yield traits, such as Sakha 105 × IRBL9-W and Sakha 101 × IRBL5-M, which gave the highest values (62.1 and 61.5, respectively). Heatmap analysis data (Fig. 3) observed the significant differences among genotypes in the studied traits (Fig. 3). Moreover, the heatmap analysis classified the genotypes based on their mean performance and their response to blast reaction. The figure showed that only two susceptible varieties were grouped and other genotypes were resistant.

Correlation analysis of the nine studied traits revealed that there was negative correlation between blast reaction (LBR) and D, PW, and 1000 GW, respectively (Fig. 4).

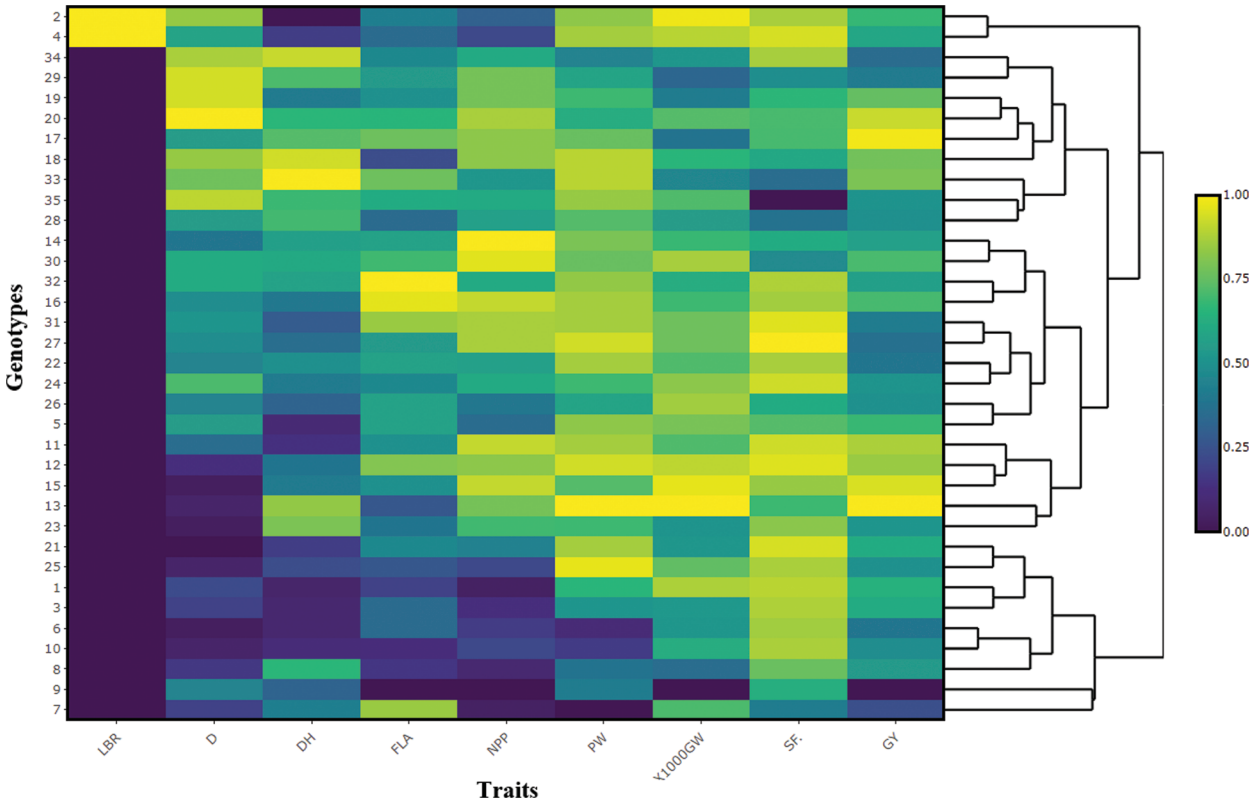

Figure 3: Heat map analysis of nine traits across 35 genotypes

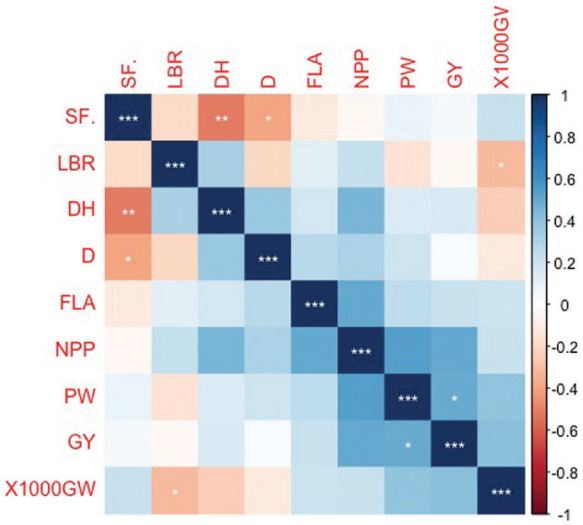

Figure 4: Corrplot depicting Pearson's correlation among nine traits across 35 genotypes. Red squares indicate a negative correlation; blue squares indicate a positive correlation; and white squares indicate no correlation. The asterisks indicate significant correlations using a two-tailed *t*-test (* and ** = $P < 0.05$; and *** $P = < 0.01$)

The present finding was also supported [36,37]. On the other hand, clustering analysis confirmed the results and divided the genotypes into two groups. The first one included all the susceptible genes, while the second one included the resistance genes. The ordinary analysis of variance for lines, tester and line × tester were shown to be highly significant mean squares for all studied traits, which indicated overall differences among the lines, tester and line × tester (Table 5). These results are in agreement with [38,39].

3.5 Phenotypic Traits and Their Segregation in the F_2 Population

Twenty-five segregation F_2 populations are presented in Table 7, and the results showed that eight crosses showed a segregation ratio of 15 (R) to 1 (S).

Table 7: Mode of inheritance in F_1 crosses and F_2 populations, χ^2 test for blast incidence

Crosses	F_1	Number of plants in F_2	Reaction types (F_2)	Expected ratio	χ^2	*P*-value
			R:S			
Sakha 105 × IRBLKS-F5	R	269	252:18	15:1	1.180	0.229
Sakha 105 × IRBL5-M	R	189	189:0	1:0	-	-
Sakha 105 × IRBL9-W	R	201	201:0	1:0	-	-
Sakha 105 × IRBLTACP1	R	153	153:0	1:0	-	-
Sakha 105 × IRBL9-W(R)	R	170	170:0	1:0	-	-
Sakha 101 × IRBLKS-F5	R	262	149:13	15:1	1.200	0.227
Sakha 101 × IRBL5-M	R	141	129:12	15:1	1.220	0.510
Sakha 101 × IRBL9-W	R	227	172:55	3:1	1.359	0.641
Sakha 101 × IRBLTACP1	R	212	190:22	15:1	3.220	0.620
Sakha 101 × IRBL9-W(R)	R	274	253-21	15:1	0.920	0.323
Sakha 103 × IRBLKS-F5	R	220	220:00	1:0	-	-
Sakha 103 × IRBL5-M	R	138	138:1	1:0	-	-
Sakha 103 × IRBL9-W	R	147	147:0	1:0	-	-
Sakha 103 × IRBLTACP1	R	155	155:0	1:0	-	-
Sakha 103 × IRBL9-W(R)	R	122	122:0	1:0	-	-
Sakha 104 × IRBLKS-F5	R	160	139:21	13:3	3.320	0.50
Sakha 104 × IRBL5-M	R	244	233:11	15:1	1.220	0.252
Sakha 104 × IRBL9-W	R	192	128:64	3:1	1.297	0.632
Sakha 104 × IRBLTACP1	R	214	192:22	15:1	1.198	0.227
Sakha 104 × IRBL9-W(R)	R	198	185:13	15:1	1.780	0.216
Sakha 106 × IRBLKS-F5	R	155	155:0	1:0	-	-
Sakha 106 × IRBL5-M	R	128	128:0	1:0	-	-
Sakha 106 × IRBL9-W	R	133	133:0	1:0	-	-
Sakha 106 × IRBLTACP1	R	95	95:0	1:0	-	-
Sakha 106 × IRBL9-W(R)	R	122	122:0	1:0	-	-

This indicates the presence of two resistance genes to leaf blast segregating in these crosses, and each gene can express resistance in the genetic background. In addition, each parent in these crosses contained one of

these genes, and the allelic relationship was complete dominance. On the other hand, two cross gave a segregated ratio of 3 (R) to 1 (S), which indicated the presence of one dominant major resistance gene transferred from these resistant parents to their offspring that controlled the resistance against blast (Table 7). Concerning the second cross, the expected ratio of 13:3 and the percentage of type reaction 139 (R) % and 21% (S) %, respectively, with *P*-value (0.10–0.50), indicated the presence of two complementary dominance genes in these crosses. On the other hand, the last one contained fourteen crosses that were found to be resistant in F_1 and all the F_2 plants were resistant without segregation, which indicates that the resistance genes in those parents could be the same or allelic. Finally, phenotypic segregation in F_2 was used to study the inheritance of resistance in offspring, which will help plant breeders in the process of resistance. However, the segregation ratios were depending for the number of genes controlling these traits such as [15 (R) to 1 (S)] means that there are two resistance genes and each gene can express resistance. While, 3 (R) to 1 (S) indicated the presence of one dominant major resistance gene transferred from these resistant parents to their offspring that controlled the blast resistance [40,41]. Also, two complementary dominance genes were found in some crosses and clarified the ratio of 13:3 [42,43].

3.6 Molecular Analysis

3.6.1 Identification of Blast Resistance Genes in the Parental Lines

Three specific markers (K3959, JJ817 and YL153/YL154) were used to identify blast resistance genes in the ten parents. Those markers are linked to *Pik-s*, *Pi 5* and *Pita-Pita-2* genes, respectively. The results as in Fig. 5 showed that the marker K3957 was linked to the *Pik-s* gene that is harbored in the IRBLKS-F5 monogenic line. This monogenic line was used as a control to check the presence of *Pik-s* gene in the Egyptian varieties. In any case, the results showed that the three Egyptian varieties (Sakha 105, Sakha 103, Sakha 106) and IRBLKS-F5 were positive to *Pik-s* gene (Fig. 5A) and two alleles (size 450 and 600 bp) were detected. While, was these were absent in Sakha 101, Sakha 104, IRBL5-M, IRBL9-W, IRBLTACP1 and IRBL9-W(R) genotypes. As for JJ817 marker, which linked with *Pi 5* gene, the monogenic line IRBL5-M was carrying this gene and was used as a donor for *Pi 5* gene in this study. The results as in Fig. 5B showed that *Pi 5* gene was present in Sakha 103 and Sakha 104 parents and absent in the rest of the genotypes.

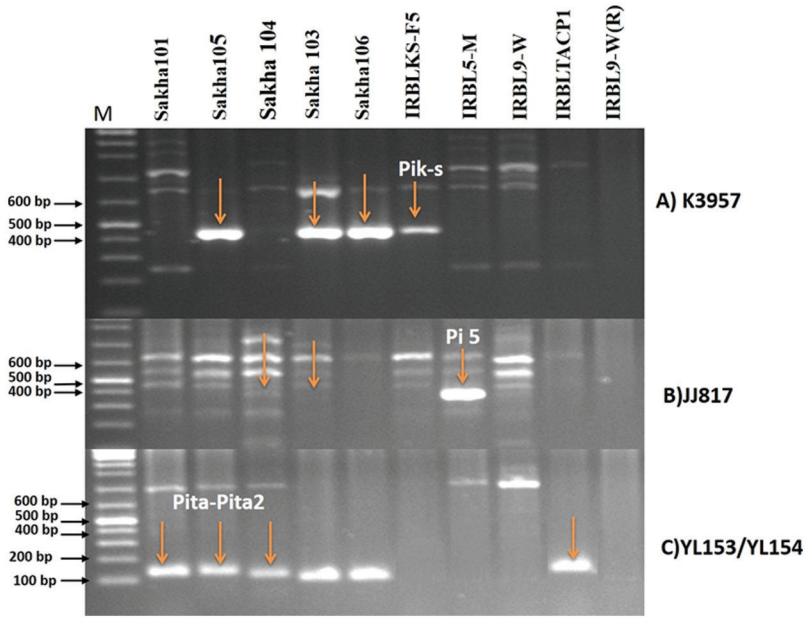

Figure 5: Amplification pattern of markers. A) K3957, B) JJ817and C) YL153/YL154, M: 100 bp DNA ladder, 1–10: denotes 10 rice genotypes included in the study, arrows indicate the specific amplified alleles

Although *Pi 5* resistant gene is detected in Sakha 104 while it was susceptible to rice blast infection in the field evaluation. This means that this gene may have no effect under the Egyptian conditions or there are any other gene(s) is affecting the expiration of this gene. Similar results were obtained with previous studies [44–47]. Positive amplified fragments for YL153/YL154 marker that linked to *Pita-Pita-2* gene were detected in three Egyptian genotypes (Sakha 105, Sakha 101, and Sakha 104) and IRBLTA-CP1 monogenic line. This monogenic line was used as a donor for *Pita* gene (Fig. 5C). On the other hand, this gene was absent in other four monogenic lines {IRBLks-F5, IRBL5-M, IRBL9-W *Pi 5(t)* and IRBLz5-CA (R)}. There are many studies that showed the importance of identifying the blast resistance genes, which helped in rice breeding programs for resistance [48–50].

In any case, this study showed the presence of *Pita* gene in cultivar Sakha 101, which was susceptible to blast disease in the field. This means that this gene is inactive under Egyptian conditions or maybe working alone to show resistance in Sakha 101. Whereas, Sakha 105 and Sakha 103 were resistant and the results showed the presence of *Pik-s* gene in those two varieties, explaining that the action of this gene is through its presence in genetic groups or that it works in complementary genes. Some of these genes were also found to be highly effective against rice blast either under Egyptian conditions [15,16] or under Chinese conditions [18].

3.6.2 Genetic Analysis in F_2 Population

Six populations and four markers were used to confirm the expected ratio and study the inheritance of blast resistance trait. The populations namely; Sakha 101 × IRBLKS-F5, Sakha 101 × IRBL5-M, Sakha 101 × IRBLTACP1, Sakha 104 × IRBL9-W(R), Sakha 104 × IRBL9-W and Sakha 104 × IRBLTACP1. The result in Table 8 showed that all the population produced 15 positives: 1 negative band with the markers JJ81-T3, JJ113-T3, RM3843 and T8042 (Fig. 6). Except for the population Sakha 104 × IRBL9-W the ratio was 3 positive: 1 negative with all the primers [51–56]. However, the data for F_2 generations were categorized into two groups according to segregation ratios. The first group, was segregated in a ratio of 15 (R) to 1 (S). The ratio 15 (R) to 1 (S) suggested that the two genes of leaf blast resistance were segregating in this cross. In addition, the same data was found in molecular markers and it confirmed that two genes were controlled in blast resistance in this cross. On the other hand, the second group of the F_2 population showed segregation of 3 (R) to 1 (S). This indicated that the resistance was transferred from resistant varieties that carried one major gene for resistance to blast disease. These results were in agreement with those of [11,16].

Table 8: Six F_2 populations produced from the crosses between resistance and susceptible parents, expected ratio and four markers

Markers (gene names)	JJ81-T3 (*Pi3(t)*)		Expected ratio	JJ113-T3 (*Pii(t)*)		Expected ratio	RM3843 (*Pi39*)		Expected ratio	T8042 (*Pit*)		Expected ratio
	1	0		1	0		1	0		1	0	
F2 populations												
Sakha 101 × IRBLks-F5	22	3	15:1	21	3	15:1	20	2	15:1	23	2	15:1
Sakha 101 × IRBL5-M	21	4	15:1	21	4	15:1	22	3	15:1	20	4	15:1
Sakha 101 × IRBLTACP1	22	2	15:1	15	2	15:1	17	4	15:1	13	1	15:1
Sakha 104 × IRBL9-W(R)	20	3	15:1	19	4	15:1	21	4	15:1	22	3	15:1
Sakha 104 × IRBL9-W	15	3	3:1	18	5	3:1	20	5	3:1	12	3	3:1
Sakha 104 × IRBLTACP1	22	3	15:1	20	5	15;1	21	4	15:1	22	3	15:1

Note: 1 = positive band for expected size, 0 = negative band for expected size.

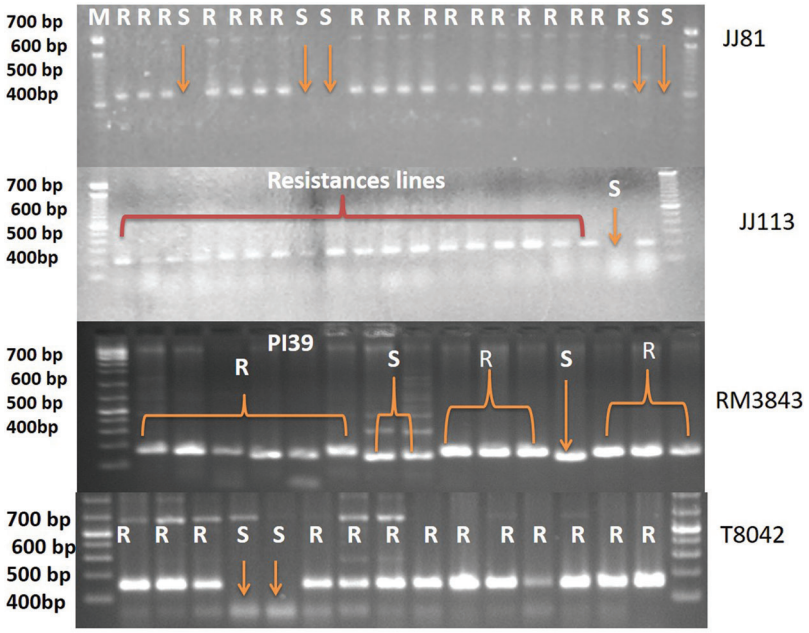

Figure 6: PCR products of gene markers JJ81-T3, JJ113-T3, RM3843 and T8042 for F_2 segregations produced from some crosses R = resistance line and S = susceptible line

In conclusion results of segregation ratio in group one (15 (R) to 1 (S)) in the F_2 generations suggested that resistant varieties carried two dominant resistance genes for leaf blast (e.g., A and B), while the susceptible variety carried their recessive alleles. While, in the second group, which showed a segregation ratio 3 to 1 in F_2 the data suggested that the genetic constitution of resistance monogenic lines could be carried by one dominant gene (AAbb). The current study clarified the importance of using the monogenic lines that are resistant to blast and use to produce and improve new varieties through the traditional breeding methods. Identification of blast resistance genes are very important in breeding program to improvement new resistance varieties [57,58]. Monogenic lines were produced to help the breeders for identifying and transfer resistance genes by conventional breeding method. In any case, there are two types of resistance: vertical resistance, which is controlled by a large number of genes with a minor effect, and horizontal resistance, which is controlled by a small number of genes with major effect. The fluctuation in effective resistance genes from one season to another and between this study and other studies may be due to the prevalence of common physiological races in every season and every location, this is agreeable by many investigators. Also, the described this phenomenon in different countries [59–61] and observed that virulent strains existed for all the identified genes of vertical resistance and most of the strains possessed virulent genes which were not necessary for their survival. In any case, Tables 3 and 4 explained the same differences between the locations under study and also the different degrees of infection between the races or strains under artificial inoculation, which confirms the theory of gene for gene.

4 Conclusion

Thirteen genes for blast were resistant under this study and the most effective genes under both natural and artificial inoculation were *Pi-Z* and *Pi-I* genes under field conditions. While, under artificial inoculation, seven genes showed complete resistance to all isolates and gave 100% resistance. *Pii*, *Piz*, and *Piz-5 = (bi2) (t)*, *Pita = (Pi4) (t)*, *Pita*, *Pib*, and *Pi1* are the completely resistant genes. The genotypes IRBLKS-F5 and IRBLTACP1, Sakha 103 and Sakha 105 varieties were the earliest varieties among the lines. The best combinations were Sakha 103 × IRBLKS-F5, Sakha 105 × IRBL9-W (R), Sakha 103 × IRBL9-W and

Sakha 103 × IRBL9-W. Different variation was also noted among the F_2 population and identification of different resistance genes against blast is an effective way of improving the resistance of rice varieties. Four rice varieties namely; Sakha 105, Sakha 103, Sakha 106 and IRBLKS-F5 carry *Pik-s* gene. While, Pita-Pita^2 gene the results showed positive amplification in the five Egyptian genotypes; Sakha 105, Sakha 101, Sakha 103, Sakha 104 and Sakha 106. The genotypes could be used for gene pyramiding and gene accumulation to produce durable resistance to blast.

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