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Molecular Profiling for Blast Resistance Genes Pita2 and Pi2/Pi9 in Some Rice (*Oryza sativa* L.) Accessions and Selected Crosses

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Abstract: Identifying major blast resistance genes in *Oryza sativa* L. genotypes is key to enhancing and maintaining the resistance. Observing rice varieties with durable resistance to blast has become a potential target in rice breeding programs. In this study, an evaluation was conducted during 2020 and 2021 on ten Egyptian and introduced varieties. First, a field experiment was conducted in a randomized complete block design with three replications, and it was found that the Egyptian cultivar Sakha 101 had the highest crop grain yields (53.27 g). The Spanish varieties Hispagrán and Puebla were the earliest (110 and 108 days, respectively) as well as the highest in 1000-grain yield, giving them priority as donors for these traits; however, they had the lowest mean values in the number of panicles. Second, these cultivars were subjected to eighteen isolates from five strains of *Pyricularia oryzae* (IH, IC, ID, IE, and II). The Egyptian varieties Giza 177, Giza 179, Sakha 106, Giza 182, GZ1368-5-5-4, and GZ6296 were 100% resistant, while Hispagrán's resistance was 16.6%, followed by Sakha 101 with 27.8%. To gain insight into the ten varieties, we used STS, SCAR, and CAPS markers to detect and mine alleles for major blast broad-spectrum resistance genes Pi2, Pi9, and Pita2. In the context of considering gene pyramiding as an effective strategy for achieving broad durable spectrum resistance to blast, molecular profiling was also conducted on eighteen F2 single plants obtained from the hybridization of Giza 177 (resistant) × Puebla (susceptible) varieties. Also, eighteen F2 single plants were obtained from Giza 177 × Hispagrán (highly susceptible) varieties. Conducting a molecular scan with STS dominant marker YL153/YL154 was performed on ten cultivars to detect the presence of the Pita2 gene, which conferred a unique band in Puebla. By doing a scan of the 18 second-generation plants derived from Giza 177 × Puebla, 11 individual plants of the 18 plants obtained a band, which was transferred from Puebla. F2 plants obtained from Giza 177 × Puebla amplified with CAPS marker RG64-431/RG64-432 had higher numbers of Pi2 alleles, while F2 plants of Giza 177 × Hispagrán cross-amplified with SCAR marker linked to Pi9 exceeded their parents more. Our results have revealed that molecular markers played an essential role in determining the direction of evolution for blast resistance traits.

Keywords: rice; blast disease; resistance; R-genes



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1. Introduction

Rice blast developed a high evolutionary rate due to a high level of instability in the genome, causing 70–80% tremendous yield losses to rice, which is considered the first nutrition source in developing countries [1]. *Magnaporthe oryzae*, the causal fungus, can infect all parts of the rice plant, including leaves, leaf collars, necks, panicles, pedicels, seeds, and roots [2,3]. Conventional breeding for durable resistance cultivars is still the primary straightforward, cost-effective, and environmentally safe method for maintaining blast resistance. Moreover, breeders have developed many blast-resistant cultivars adapted to different rice-growing regions worldwide. However, durability resistance is still a challenge facing rice breeders [4].

Resistance breakdowns, such as Reiho resistance lost during its first-year production in Egypt, result in a serious epidemic [5]. There is an indispensable need for broad-spectrum disease resistance, defined as the resistance to the majority of geographically different races or isolates of the same pathogen [6–8].

Plants produce resistance genes that can directly or indirectly recognize the pathogen's secreted effectors to initiate a second defense called effector-triggered immunity (ETI), culminating in hypersensitive cell death [9–11]. More than 100 major R-genes have been identified and mapped [12,13]. Chromosomes 6 and 12 in rice are distinguished by containing elite blast resistance genes that are fundamental in resistance architecture [14,15]. Broad-spectrum R-genes Pita and Pita2 are located on chromosome 12 and a minimum of eight other genes [16,17]. Pita2 encodes R proteins differently from Pita and conferred a broader resistance spectrum [18–20]. Also, Pita2 shows resistance to all Pita virulent isolates and some Pita virulent isolates [21,22]. In addition, chromosome 6 indicated that the Pi2/Pi9 is situated close to the centromere 6 [23], and the Pi2/Pi9 locus includes Pi2, Pigm, Piz-t, and Pi9 [24]. The Pi9 gene of a nucleotide-binding site leucine-rich repeat (NBS–LRR) family is considered a key point in elucidating the molecular basis of the broad-spectrum disease resistance [6].

Previous studies illustrate the importance of the clustered arrangement of R-genes in association with the evolution of novel resistance specificities exerted by pathogen populations throughout evolutionary history [23,25]. So far, thirteen Pi9 alleles have been identified that are varied in their blast-resistance spectra. These variations are the main reason for the durability of Pi9 against *Magnaporthe oryzae* [26]. Polymerase chain reaction (PCR) based markers have been developed to screen different blast R-genes. DNA markers represent an important tool for increasing the precision of the identification of blast resistance genes in a breeding program [27–29].

Several Egyptian commercial rice varieties with broad-spectrum resistance have been used as sources of resistance; but R-genes have not been characterized. This study aimed to (1) evaluate the crucial traits in ten genotypes in order to demonstrate their phenotypic architecture and survey the genetic advance, variance, and heritability; (2) screen leaf blast resistance reaction under blast nurseries (natural infection) and greenhouse (18 isolates) to understand how rice blast is affected by environmental conditions; (3) examine R-genes (Pi2, Pi9, and Pita2) in rice genotypes and F2 plants.

2. Materials and Methods

2.1. Field Experiment

The present investigation was conducted at the experimental farms of the Rice Research and Training Centre located in Sakha, Kafr El Sheikh, during 3 seasons in 2020, 2021, and 2022.

The genetic materials used in this investigation involved ten Egyptian commercial varieties and promising lines as well as two introduced varieties (Table 1). During the

summer seasons of 2020 and 2021, the experiment was conducted in a randomized complete block design (RCBD) with three replications. The plants were grown in three rows with a spacing of 20 cm between the rows and 15 cm between the plants. The experiment was conducted according to the agricultural practice recommended for the region. Fertilizers were added at the recommended rates and time of application: 15 days after transplanting at the rate of 40 kg N/fed and 15 kg P₂O₅/fed. Hand weeding was done.

Table 1. Pedigrees, origin, type, and year of release of the selected genotypes.

Genotype	Pedigree	Origin	Type	Year of Release
Giza 177	Giza171/Yu ji No.1/pi No.4	Egypt	Japonica	1995
Giza 179	GZ6296/GZ1368	Egypt	Indica/Japonica	2013
Giza 182	Giza 181/IR 39422–163-1-2/Giza 181	Egypt	Indica	1999
Sakha 101	Giza 176/Milyang 79	Egypt	Japonica	1997
Sakha 104	GZ4096-8-1/GZ4100-9-1	Egypt	Japonica	1999
Sakha 106	Giza 177/Hexi 30	Egypt	Japonica	2010
GZ1368-5-5-4	Not available	Egypt	Indica	Promising line
GZ6296-12-1-2-1-1	GZ 7185 -7-4-3-1/Nonjing	Egypt	Indica	Promising line
Puebla	Not available	California (USA)	Japonica	Introduced variety
Hispagrán	Not available	California (USA)	Japonica	Introduced variety

2.2. Studied Characters

The studied characters (quantitative parameters) were recorded for ten rice varieties grown under flood irrigation. The studied characters were the number of days to maturity, number of panicles per plant, 1000-grain weight (g), and grain yield per plant (g) according to IRRI [30]. Phenotypic and genotypic coefficients of variation (PCV and GCV) for all the studied characters were calculated by the formula reported by Burton [31]. The genetic advance as a percentage of the mean (GAM) and broad-sense heritability for all the traits as the ratio of GCV to the total PCV were calculated as reported by Johnson et al. [32].

2.3. Data Analysis of the Genetic Parameters

The data were analyzed using the ordinary analysis of variance to test the significance of differences among the studied genotypes using the “MSTAT” computer software package version 5.4.

2.4. Blast Disease Infection

In 2022, the cultivars in the blast nurseries of Sakha and Gemmiza were evaluated from 1st June. The cultivars were planted in seedbeds and fertilized with 60 Kg N/fed and farmyard manure at a rate of 8 m³/fed. The plots of the seedbeds were 1 meter wide and 10.5 m long. Susceptible check varieties were planted after every 10 lines of the cultivars as a spreader line and also planted throughout the border of the blast nursery as a borderline. The susceptible checks followed by one row of the resistant checks were sown alternatively, surrounding the cultivars. Then, they were left exposed to natural blast infection at the seedling stage. About 40 days from sowing, the blast lesions were scored from 0 to 9.

Greenhouse infection was conducted in the same season (2022) with the use of 18 isolates of *Pyricularia oryzae* obtained from Sakha 101 and Sakha 104 from six rice-growing governorates. Blast-infected leaves were collected from the old rice field. First, the leaf samples were washed with sodium hypochlorite and then in distilled water before being incubated in Petri dishes at 32 °C to enhance sporulation. After incubation, single spores were picked under a microscope with a pin-loop and placed on a banana medium. The fun-

gal growth started within 7 days after spore placement on the medium [33]. Pathogenicity tests and race identification studies were carried out for the obtained fungal isolates under greenhouse conditions with artificial infection. International differential varieties (I.D.V.) were used to identify blast physiological races. Genotypes were seeded in plastic trays (30 × 20 × 15 cm). Each tray comprised 10 rows; out of them, 2 rows were cultivated with susceptible rice checks. The trays were kept in the greenhouse at 25–30 °C and fertilized with urea (46.5% N; 5 g/tray). Seedlings at the 3–4-leaf stage (about 3–4 weeks after sowing) were inoculated with spore suspension (100 mL) at 5×10^4 spores/mL using an electrical spray gun. The inoculated seedlings were held in a moist chamber with at least 90% relative air humidity and 25–28 °C temperature for 24 h and then moved to the same conditions in the greenhouse. Each plant was scored for rice blast disease severity on a 0 to 9 scale [34].

2.5. F2 Seeds

Mating was carried out for making two different crosses: the first cross Giza 177 and Puebla, and the second cross Giza 177 and Hispagrán. In the flowering period, the bulk emasculation method was applied according to Jodon [35] and modified by Butany [36], using hot water (42–44 °C for 10 min). Plants were taken from F1 and advanced to F2. Eighteen F2 single plants from each cross were subjected to extract DNA to show the inheritance of blast resistance genes.

2.6. DNA Extraction

In 2022, genomic DNA from ten genotypes and eighteen F2 single plants Giza 177 (resistant) × Puebla (susceptible) and eighteen F2 single plants Giza 177 (resistant) × Hispagrán (highly susceptible) was extracted and quantified from three-week-old immature tender leaves, according to a modified CTAB method published by Murray and Thompson [37]. The extracted DNA was kept at −20 °C. A spectrophotometric technique and agarose gel electrophoresis were used to confirm and quantify the extracted genomic DNA. Three markers (STS and CAPS, SCAR markers) were used in this study, as shown in Table 2. The primer sequences were directly downloaded from the Gramene website (www.gramene.org). PCR reactions were carried out in 20 µL volume containing 2.0 µL of DNA ($15 \text{ ng } \mu\text{L}^{-1}$), 6.0 µL of dd.H₂O, 10.0 µL of 10 × PCR master max buffer, 1.0 µL forward primer ($30 \text{ ng } \mu\text{L}^{-1}$), and 1.0 µL reverse primer ($30 \text{ ng } \mu\text{L}^{-1}$). The reaction mixture was subjected to the following conditions: initial denaturation at 94 °C for 5 min, and then 35 cycles of amplification under the following parameters: template denaturation at 94 °C for 1 min, primer annealing at 50–62 °C for 24 s, and extension at 72 °C for 2 min. By the end of the 35th cycle, a final extension at 72 °C for 10 min was given, followed by storage at 4 °C. PCR thermocycler machines from Biometra and Applied Biosystems were used. DNA bands were separated on 3% agarose gel [38] and stained with ethidium bromide, and the band size was measured and analyzed by myImage analysis software version 1.1.753.

Table 2. Marker names, chromosome location (CL), molecular size (MS), annealing temperature (AT), and sequences for the DNA primers used in PCR technique.

Marker Name		R-Gene	CL	MS (bp)	AT (°C)	Forward Sequence	Reverse Sequence	Reference
STS	YL155/YL87	Pita2	12	1040	59	5'-caacaatttaatacatcacg-3'	5'-atgacacccctgcgatgcaa-3'	[39]
CAPS	RG64-431/ RG64-432	Pi2	6	1400	61	5'-Gttgtttgagctctccaatgcctgttc-3'	5'-Ctgcagtgcaatgtgcggccagg-3'	[40]
SCAR	PBA12	Pi9	6	400	60	5'-Tggtgcactcagaaagaa-3'	5'-Gcagtgctatctgtctcc-3'	[39,41]

3. Results and Discussion

3.1. Means of Performance

Phenotypic evaluation (Table 3) was conducted over ten rice genotypes. From means of days to maturity the remarkable varieties that achieve the latest period from seed to seed, consequently, may help in escaping disease and may be a smart strategy for facing the water shortage challenge. Thus, these genotypes are intrinsic in breeding programs. The varieties were categorized into three groups: the first earliness group includes Puebla and Hispagrán, which obtained the lowest mean values of 108 and 110 days, respectively. The second group contains days to maturity ranging from 123 to 135 days, whereas the third group spends the longest time reaching maturity at 136 and 143 days in Sakha 104 and Sakha 101, respectively. Draz et al. [42] reported that Sakha 101 exhibited the highest mean value for days to maturity (140 days).

Table 3. Mean performances of the studied genotypes.

Genotypes	Days to Maturity	Number of Panicles per Plant	1000-Grain Weight (g)	Grain Yield per Plant (g)
Giza177	123	19.00	26.17	41.16
Giza179	124	20.11	27.14	43.57
Giza182	126	22.67	23.96	40.83
Sakha 101	143	24.92	28.70	53.27
Sakha 104	136	24.11	26.86	51.55
Sakha 106	127	19.44	28.16	40.25
GZ1368-5-5-4	135	23.33	23.87	44.28
GZ6296-12-1-2-1-1	135	20.56	26.82	43.43
Puebla	108	15.04	34.20	40.86
Hispagrán	110	17.06	39.24	45.13
LSD at 5%	0.04	0.05	0.10	0.06
LSD at 1%	1.00	1.02	1.11	1.06

Concerning the number of panicles per plant, results in Table 3 clearly show that Sakha 101 recorded the highest mean performance (24.92), and the second highest mean value was scored by Sakha 104 (24.11), followed by GZ1368-5-5-4 (23.33) and then Giza 182 with 22.67. On the other hand, the Spanish varieties Puebla and Hispagrán recorded the lowest number of panicles per plant 15.04 and 17.06, respectively. Giza 177, Sakha 106, Giza 179, and GZ6296-12-1-2-1-1 gave medium mean values (19.00, 19.44, 20.11, and 20.56, respectively).

The 1000-grain weight of Spanish varieties ranked the most desirable mean values as 34.20 g and 39.24 g for Hispagrán and Puebla, respectively, whereas the Egyptian varieties showed medium to low performance ranging from 23.87 g to 28.70 g for GZ1368-5-5-4 and Sakha 101, respectively. Grain yield is the final and most essential parameter, and higher yield potential is the breeder's ultimate goal in developing countries. The highest grain yield per plant ratio was in Sakha101 and Sakha 104 were recorded at 53.27 g and 51.55 g, respectively. Abd Allah et al. [43] also reported that Spanish and Delta-Med genotypes generally had low grain yield, shorter duration, and higher 1000-grain weight.

3.2. Analysis of Variance for Varieties and Parameters

The phenotypic variance was combined genotypic variance with the environment. It was found to be high for days to maturity (132.22), low for the number of panicles per plant (13.44), and intermediate for 1000-grain weight (27.49). Genetic variance, which refers to three major components, additive, dominance, and epistasis, showed wide variability among the genotypes. Days to maturity obtained the highest genetic variability, 131.60 (Table 4), whereas low genetic variance was detected for the number of panicles per plant

(12.17). There was a direct relation between the phenotypic coefficient of variation (PCV%) and the genotypic coefficient of variation (GCV%). The highest PCV% and GCV% values were found for 1000-grain weight, whereas the lowest PCV% and GCV% were found for the number of days to maturity.

Table 4. Estimates of phenotypic variance (PV), genetic variance (GV), phenotypic coefficient of variation (PCV), genetic coefficient of variation (GCV), genetic advance (GA), and broad-sense heritability (h^2) in a broad sense for all studied characters for selected genotypes.

Characters	PV	GV	PCV (%)	GCV (%)	GA	GA%	h^2_{bs} %
Days to maturity	132.22	131.60	9.14	9.12	23.58	18.74	99.53
Number of panicles per plant	13.44	12.17	17.97	17.10	6.84	33.50	90.56
1000-grain weight (g)	27.49	25.21	18.32	17.55	9.91	34.61	91.71
Grain yield per plant (g)	27.09	26.16	11.68	11.47	10.35	23.23	96.56

Concerning genetic advance (GA), a reliable meaningful parameter that expresses the genetic gain and couples with heritability, the highest values were in days to maturity (23.58). On the other hand, the lowest value was recorded for the number of panicles per plant (6.84). Meanwhile, the genetic advance percentage (GA%) represented high values for the 1000-grain weight (34.61%), followed by the number of panicles per plant (33.50%), and the lowest percentage was found for the number of days to maturity (18.74%).

Assessment of heritability helps breeders to allocate resources crucial to effectively select for achieving optimized genetic gain with limited resources [44]. Broad-sense heritability was high for the chosen characters; the values ranged from 90.56 to 99.53%. The highest percentage was for days to maturity, and the lowest percentage was for the number of panicles per plant.

3.3. Blast Disease Screening Under Artificial and Natural Infection

The ten genotypes were evaluated in the field under natural infection (blast nursery). The source of infection under the blast nursery came from susceptible varieties planted in the same spreader. Leaf infections were scored according to the IRRI Standard Evaluation System (1996) by using a 0–9 score [40]. As shown in Table 5, Egyptian genotypes Giza 177, Giza 179, Giza 182, GZ1368-5-5-4, and GZ6296-12-1-2-1-1 were scored as resistant [45], Sakha 101, Sakha 104, and Puebla as susceptible varieties, and Hispagrán as highly susceptible [46].

Table 5. Scores of leaf blast reaction for the studied genotypes over two locations during 2022 using a 0–9 SES scale.

Local Varieties	Leave Blast Reaction	
	Gemmiza	Sakha
Giza 177	1	1
Giza 179	1	1
Giza 182	1	1
Sakha 101	6	5
Sakha 104	5	5
Sakha 106	1	1
GZ1368-5-5-4	1	1
GZ6296-12-1-2-1-1	1	1
Puebla	4	5
Hispagrán	7	7

Eighteen blast isolates were collected from susceptible Sakha 101 and Sakha 104 cultivars from six rice-growing governorates during the 2022 season. The 18 isolates

were categorized into five races, IC, II, IH, ID, and IE, as shown in Table 6. Race virulence percentages were highest in ID, IC, and then IH, while IE showed a low virulence percentage (Figure 1).

Table 6. Rice blast isolates from different governorates, Egyptian rice cultivars, and their race identification (2022).

Governorate	District	Rice Cultivar	Race Identification
Kafrelsheikh	Sakha	SK 104	IH-1
Kafrelsheikh	Qallin	SK 101	IH-1
Gharbia	Mahala-rouh	SK 104	ID-9
Damietta	KafrSaad	SK101	ID-15
Kafrelsheikh	Alhamra	SK 101	IC-31
Kafrelsheikh	Qallin	SK 101	IH-1
Ismallea	Eltal Elkbeer	SK 104	IH-1
Gharbia	Shabsher Elhessa	SK 101	ID-12
Kafrelsheikh	Elryad	SK 104	IC-8
Kafrelsheikh	Alagozeen	SK 104	ID-11
Kafrelsheikh	Elmrabeen	SK 104	II-1
Kafrelsheikh	Sakha	Unknown	IC-1
Gharbia	Samanood	SK 108	IC-11
Dakahlia	ELmansoura	SK 108	ID-11
Kafrelsheikh	Desoq	SK 104	IC-11
Sharquia	Zagazeg	SK 101	IE-8
Kafrelsheikh	El-sheen	SK 101	II-1
Kafrelsheikh	Sakha	SK 108	IC-11

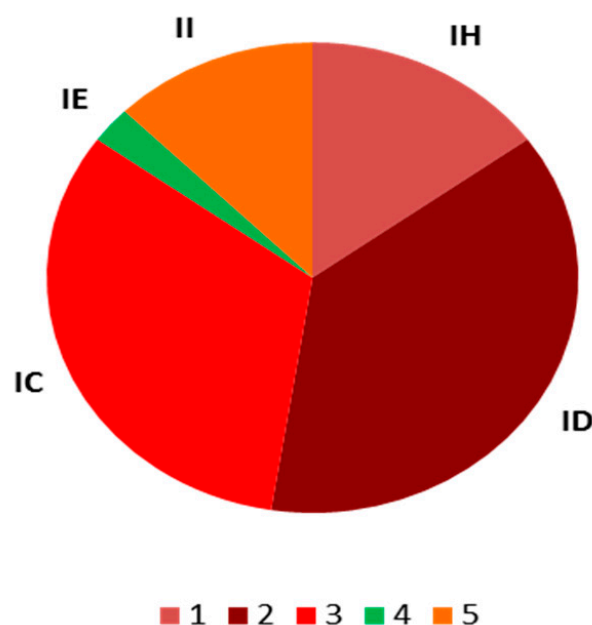


Figure 1. Races virulence percentage: (1) IH, (2) ID, (3) IC, (4) IE, (5) II.

The isolates were identified according to their reaction to international differential varieties at the race level (Table 7).

Race identification and monitoring determine race shifting; the collected data indicated that race group IC represented the most common races in 2018 and 2019 [47], but IB, IF, and IG were absent, while Sehly et al. [48] categorized the isolates into seven race groups, i.e., IA, IC, ID, IB, IG, IH, and II races.

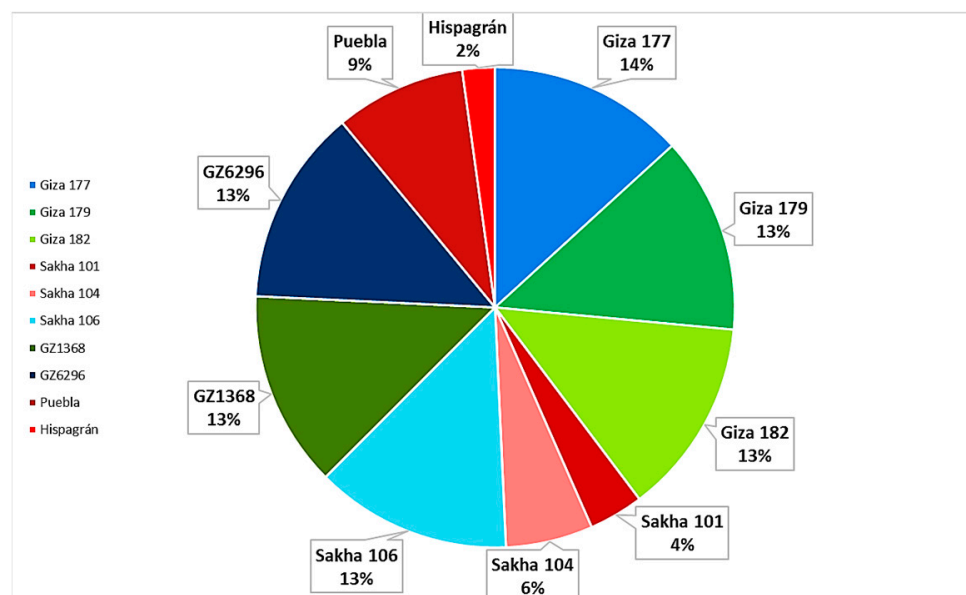
The results (Table 8, Figure 2) show that Hispagrán is highly susceptible with a resistance percentage of 16.6%.

Table 7. Levels of blast resistance genes in Japanese differential varieties (JDV) under artificial inoculation to 18 rice blast races.

JDVs	Target Gene	Isolate No./Reaction																		Resistance %
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Shin2	<i>Pi-ks</i>	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	100
Toride1	<i>Pi-zt</i>	1	1	5	8	4	3	1	4	7	1	9	4	9	1	1	1	1	1	55.6
Tusyake	<i>Pi-km</i>	1	1	1	1	8	1	4	1	1	1	9	8	1	8	1	1	1	1	72.2
Kanto51	<i>Pi-k</i>	1	1	6	4	8	8	8	1	1	1	9	8	8	1	1	1	1	4	44.4
Fukunishiki	<i>Pi-z</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Ishikari	<i>Pi-I</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Shiroke	<i>&ks</i>	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
BL-1	<i>Pi-b</i>	1	1	4	4	1	1	1	4	8	4	3	4	1	4	1	1	1	1	16.1
Yashiro	<i>Pi-ta</i>	3	1	7	8	1	1	1	4	4	7	4	5	1	4	4	4	1	1	44.4
Mochi	<i>Pi-ta2</i>	5	1	5	9	1	1	2	4	5	8	8	4	1	5	1	1	1	1	44.4
Pi No.4	<i>Pi-a</i>	5	1	1	4	1	5	9	4	4	9	4	4	1	8	1	1	1	1	50
Aichi Asahi																				
Virulence %		20	0	50	60	30	20	30	50	50	50	60	70	20	60	10	10	0	10	

Table 8. Genotypes reactions under artificial inoculation against 18 rice blast races.

Genotypes	Isolate No./Race Identification/Reaction																		Resistance %
	1 IH-1	2 IH-1	3 ID-9	4 ID-15	5 IC-31	6 IH-1	7 IH-1	8 ID-12	9 IC-8	10 ID-11	11 II-1	12 IC-1	13 IC-11	14 ID-11	15 IC-11	16 IE-8	17 II-1	18 IC-11	
Giza 177	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Giza 179	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Giza 182	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Sakha 101	4	4	4	5	1	1	1	4	4	8	8	5	1	9	6	4	4	1	27.8
Sakha 104	1	1	1	1	8	4	5	4	1	4	7	4	4	4	1	1	1	4	44.4
Sakha 106	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
GZ1368	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
GZ6296-12-1-2-1-1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	100
Puebla	1	4	4	1	1	1	1	1	4	3	4	1	4	7	1	1	1	1	66.6
Hispagrán	1	1	6	8	8	9	9	9	5	5	9	5	9	9	8	1	5	8	16.6

**Figure 2.** Blast resistance percentage of the ten rice genotypes. (1) Giza 177, (2) Giza 179, (3) Giza 182, (4) Sakha 101, (5) Sakha 104, (6) Sakha 106, (7) GZ1368-5-5-4, (8) GZ6296-12-1-2-1-1, (9) Puebla, (10) Hispagrán.

Sakha 101 was also highly susceptible with a resistance percentage of 27.7%. While Sakha 104 was susceptible, its resistance to the eighteen isolates was 44.4%, and Puebla was susceptible with a resistance of 66.6%. The other Egyptian varieties showed 100% resistance.

Figure 3 shows the leaf blast lesions formed from infection by isolate number 11 (race II-1) on the highly susceptible varieties Hispagrán and Sakha 101, susceptible variety Puebla, and resistant varieties Giza 182 and Giza 177.

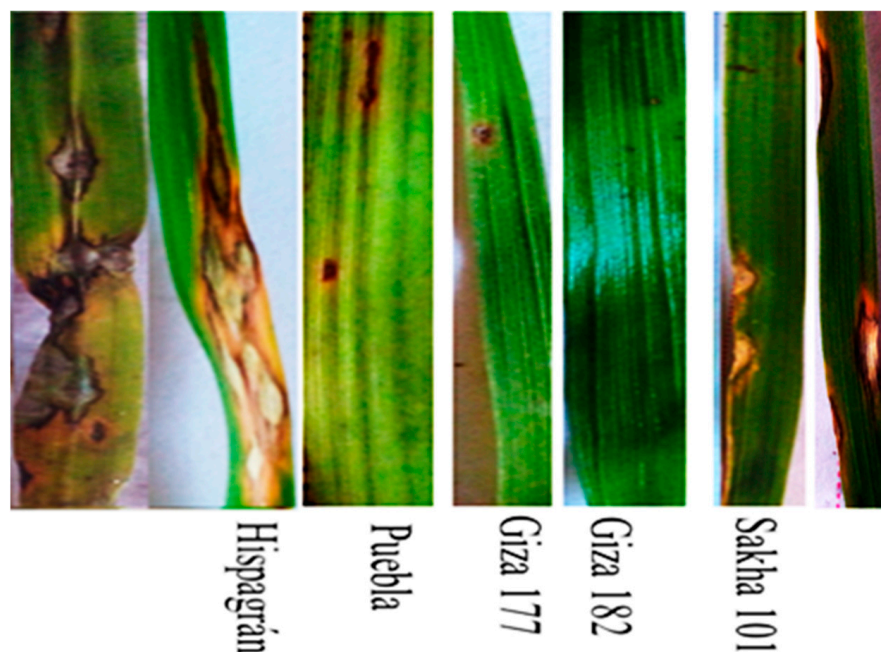


Figure 3. Leaf blast lesions on some varieties.

3.4. Blast Gene Identification Based on Markers

The selected rice genotypes and individual plants of F2 populations obtained from the crosses of Giza 177 × Hispagrán and Giza 177 × were subjected to screening blast resistance genes using DNA markers. Three primers (SCAR, CAPS, and STS markers) were used to identify blast resistance genes, as shown in Figures 4–11. The distribution of the resistance genes and their expected bands are presented in Tables 9 and 10.

The dominant molecular markers YL155 and YL187 are developed to amplify the expected bands with a molecular weight of 1040 bp for the Pita2 resistance gene [49]. The data of Figure 4 confirm the presence/absence of the Pita2 gene. The expected band (1040 bp) was polymorphic and found in one genotype, only in the susceptible introduced variety Puebla (Table 9). Notwithstanding, the gene Pita2 is absent in Giza 177 and was not inherited from its parents.

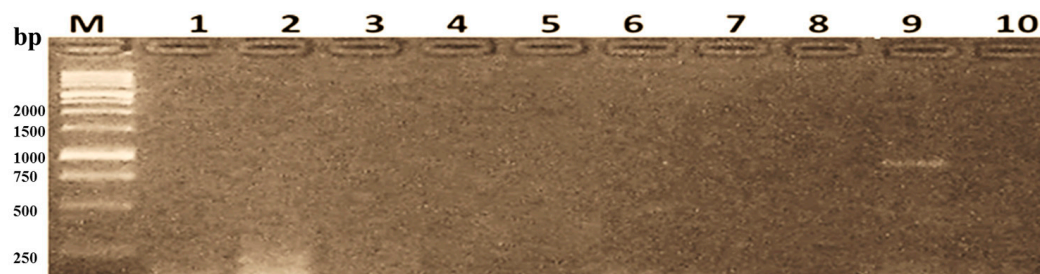


Figure 4. Screening of the ten rice genotypes under study using the dominant STS marker YL155/YL87 for Pita2; (1) Giza 177, (2) Giza 179, (3) Giza 182, (4) Sakha 101, (5) Sakha 104, (6) Sakha 106, (7) GZ1368-5-5-4, (8) GZ6296-12-1-2-1-1, (9) Puebla, (10) Hispagrán, (M) ferments as 1000 bp ladder.

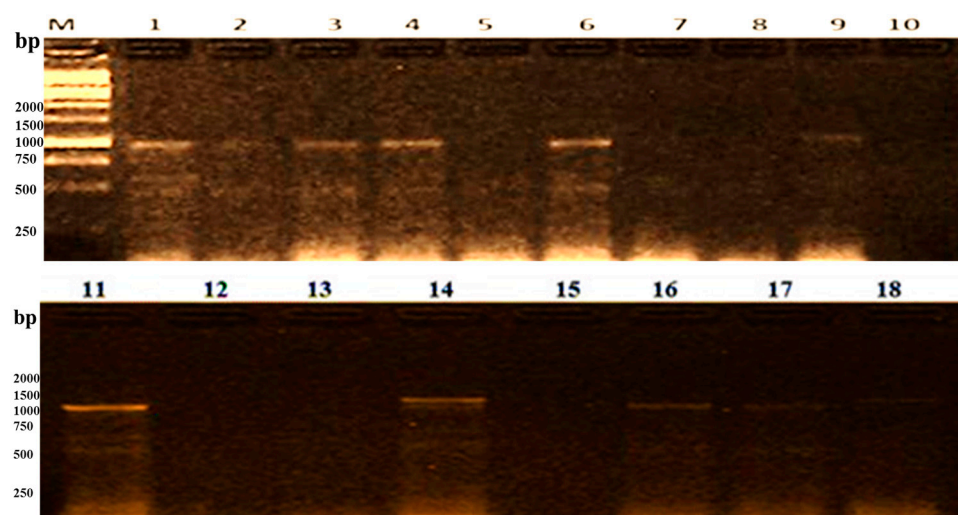


Figure 5. Screening of the eighteen F2 individual plants of the cross Puebla $\times$ Giza 177 using the dominant STS marker YL155/YL87.

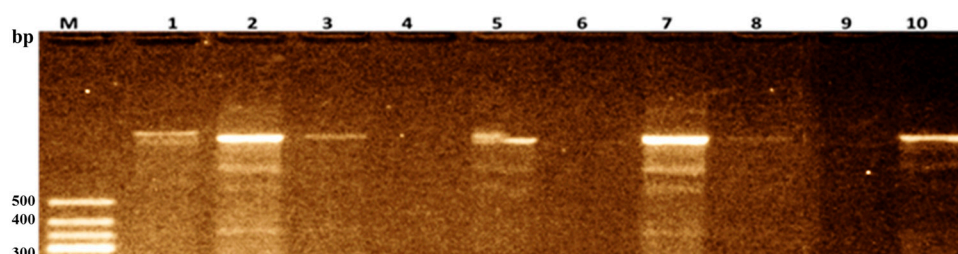


Figure 6. Screening of the ten rice genotypes under study using SCAR marker RG64-431/RG64-432 closely linked to Pi2 gene; (1) Giza 177, (2) Giza 179, (3) Giza 182, (4) Sakha 101, (5) Sakha 104, (6) Sakha 106, (7) GZ1368-5-5-4, (8) GZ6296-12-1-2-1-1, (9) Puebla, (10) Hispagrán, (M) ferments as 50 bp ladder.

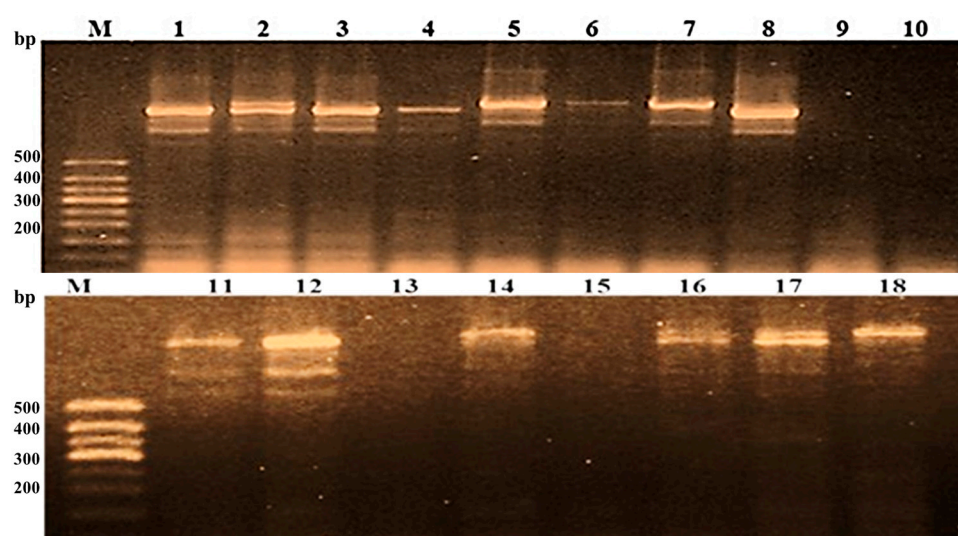


Figure 7. Screening of the eighteen F2 individual plants of the cross Puebla $\times$ Giza 177 using CAPS marker RG64-431/RG64-432.

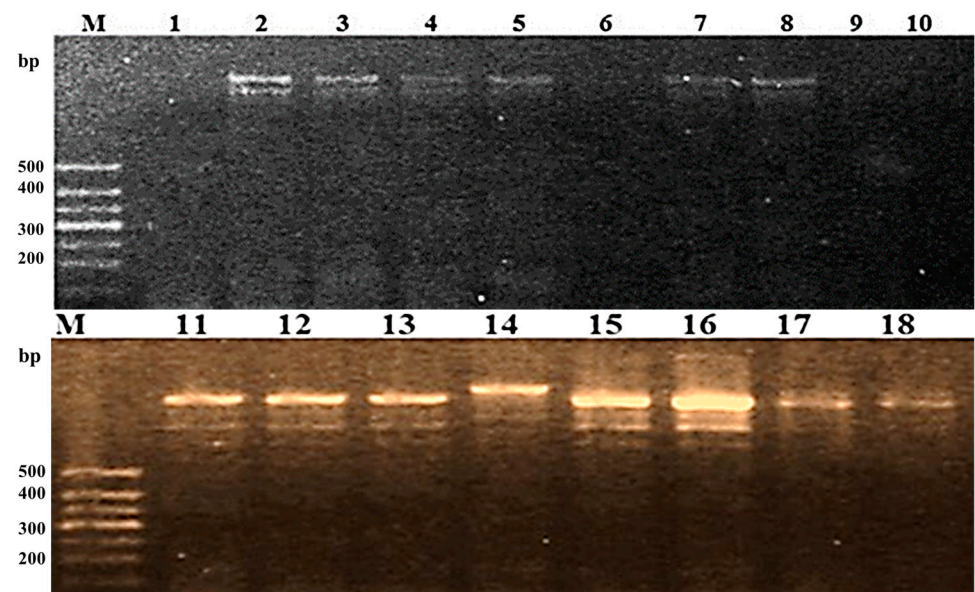


Figure 8. Screening of the eighteen F2 individual plants of the cross Hispagrán $\times$ Giza 177 using CAPS marker RG64-431/RG64-432.

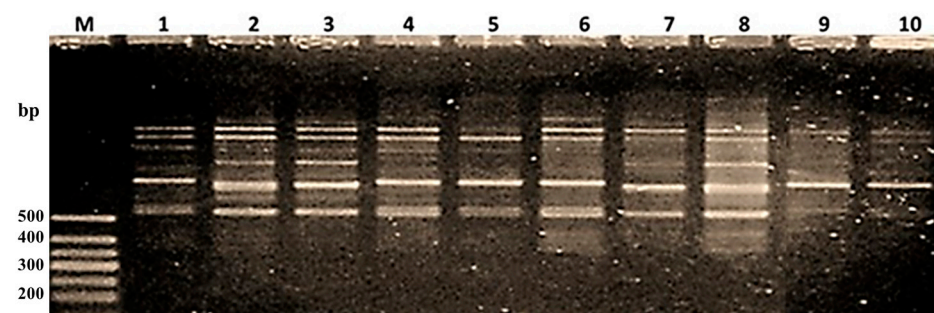


Figure 9. Screening of the ten rice genotypes under study using SCAR marker PBA12; (1) Giza 177, (2) Giza 179, (3) Giza 182, (4) Sakha 101, (5) Sakha 104, (6) Sakha106, (7) GZ1368-5-5-4, (8) GZ6296-12-1-2-1-1, (9) Puebla, (10) Hispagrán, (M) ferments as 50 bp ladder.

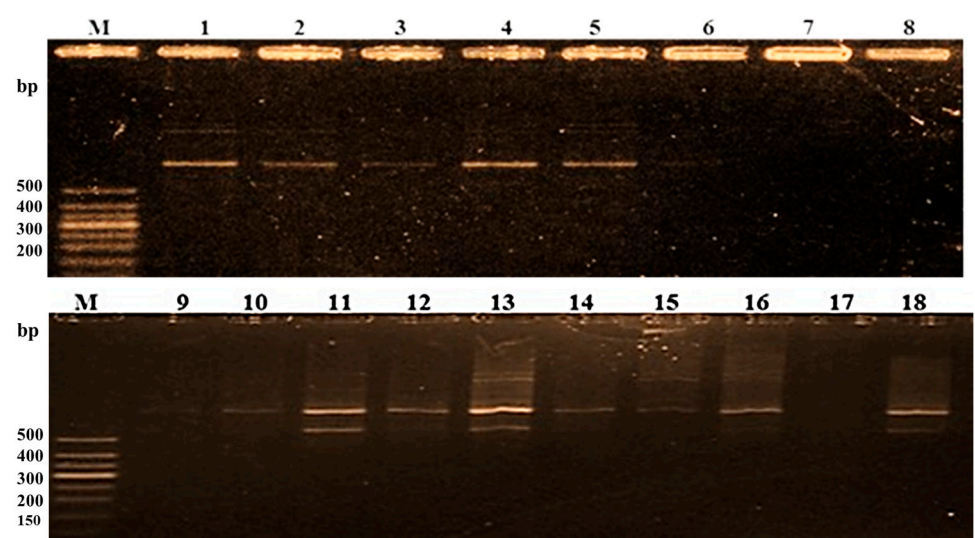


Figure 10. Screening of the eighteen F2 individual plants of the cross Puebla $\times$ Giza 177 using SCAR marker PBA12.

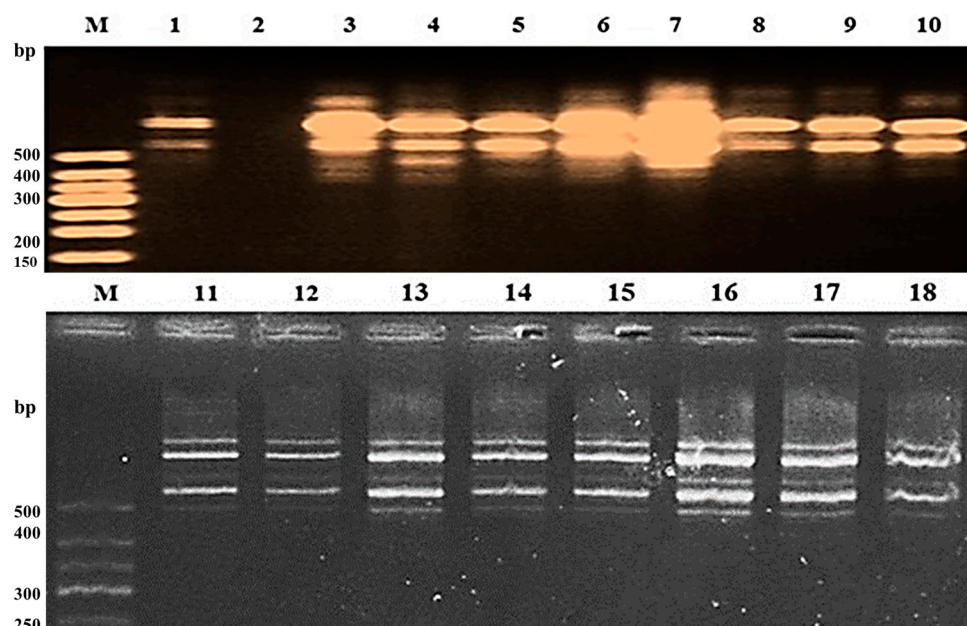


Figure 11. Screening of the eighteen F2 individual plants of the cross Hispagrán $\times$ Giza 177 using SCAR marker PBA12.

Synchronization, screening eighteen F2 plants of the cross Puebla $\times$ Giza 177, was a crucial potential to validate the presence of Pita2. The dominant allele was inherited by eleven plants, as indicated in Figure 5 and Table 10. The relationship between Pita and Pita2 was established by Meng et al. [10] in the Philippines and Burundi, who concluded that Pita2-mediated field resistance was relatively prevalent. Furthermore, reports suggest that Pita2 conferred a broader resistance spectrum than Pita [21,22]. In this study, we found that using Japanese cultivars, the resistance percentage of these genes was equal to the Pita and Pita2 genes (44%), making the gene susceptible under the conditions characteristic of 2022 in Egypt (Table 7).

Cleaved amplified polymorphic sequence (CAPS) marker RG64-431/RG64-432 is closely linked to Pi2, which is located on chromosome 6 [50]. It amplified the nine polymorphic fragments ranged from 1450 bp to 100 bp and the expected band with molecular size 1040 bp in the ten studied varieties as specified in Table 9. The desired constructive Pi2 gene pool was found in Giza 177, Giza 179, and GZ1368-5-5-4, which exhibited all nine bands, while Pi2 alleles did not exist in Sakha 101 and Sakha 106. The marker reveals only one band with the same molecular size of 1400 bp in the susceptible variety Puebla and resistant variety GZ6296-12-1-2-1-1. Among genotypes, Sakha 104 gave five bands and Hispagrán gave seven bands.

Eighteen individual F2 plants generated from Giza 177 $\times$ Puebla were screened for Pi2 (Table 10, Figure 7). The expected band with a molecular size of 1400 bp was amplified in twelve plants. The fragment with a molecular size of 250 pb was absent in all plants. Figure 7 shows that eight alleles were accumulated in one plant, and another two plants were devoid of Pi2 bands. Also, four plants carried seven bands, and two plants showed the presence of only one band. It was distinguished that no plant contained all fragments analog to Giza 177. From the standpoint of population genetics, the most elementary step in evolution is a change in gene frequency [51].

Analyzing R-genes in the F2 population has advanced our understanding of gene architecture and accumulations. Eighteen F2 Giza 177 $\times$ Hispagrán individual plants were amplified with CAPS marker RG64-431/RG64-432, as shown in Figure 8; the maximum number of amplified fragments was five. Four plants were destitute of the Pi2 allele

and only one plant gave five fragments, while thirteen plants showed the expected band (1400 bp).

F₂ plants Giza 177 × Puebla give more significant accumulation and variation of alleles of the Pi2 gene than F₂ Giza 177 × Hispagrán plants. It makes the F₂ population driven from Giza 177 × Puebla contain a better chance to have a superior allele for the Pi2 gene.

The SCAR (sequence characterized amplified region) marker is a co-dominant marker that detected twelve polymorphic bands with molecular sizes ranging from 2000 bp to 350 bp and two monomorphic bands with molecular sizes of 1400 bp and 1500 bp. The varieties that showed the expected band with a molecular size of 400 bp (Table 9, Figure 9) were Giza 179, Sakha 106, and GZ6296-12-1-2-1-1. Moreover, GZ6296-12-1-2-1-1 is an indica variety that amplified the largest number of fragments (11), so this variety is considered a genetic pool for Pi9. It is also regarded as an important source of this gene in breeding programs by hybridization to produce broad-spectrum resistant varieties to blast disease [52,53]. These results agree with Wu et al. [54], who reported that Pi9 was only present in indica-type accessions.

For Giza 177 × Puebla, eighteen F₂ plants were evaluated with a SCAR marker; the bands' patterns are shown in Figure 10 and Table 10. Accumulating five bands in one plant and two plants devoid of bands, we could conclude that all fragments did not inherit from the two parents. The majority of plants assembled one band.

The second-generation plants derived from Giza 177 × Hispagrán ranged from one individual plant containing 13 fragments to a plant devoid of fragments; these results are illustrated by Figure 11. The expected fragment with a molecular size of 400 bp was found in four plants. The difference and diversity in the fragments are evidence of the widening of the genetic base pool of this gene and increased allelic diversity within the F₂ plants resulting from a cross between a resistant father (Giza 177) and a highly susceptible father (Hispagrán), while the genetic base was less for plants produced between a resistant father and a susceptible father only (Puebla). Allele combination is of utmost importance for the utilization of genetic resources in crop improvement as well as using different genomes for efficient utilization to increase allelic diversity at the Pi9 gene in terms of improving the blast resistance trait.

Traditional breeding by introducing varieties and hybridizing is an effective way to pyramiding genes not only to face the instability of blast physiological races but also to increase the ability of the varieties to adapt to climate change and drought because these conditions increase the severity of blast disease. As the variety Puebla is susceptible under Egyptian conditions, it contains the Pita2 gene, which contributed to the evolution of the blast resistance trait.

A major goal of DNA markers is to uncover the genetic and molecular architecture that underlies phenotypes and to identify the genotypes and phenotypes that underlie adaptation to divergent environments. Genotypic diversity usually results from the addition or deletion of the entire repeat units or motifs. Therefore, different individuals exhibit variations as differences in repeat numbers. In other words, the polymorphisms observed in markers are the result of differences in the number of repeats of the motif caused by polymerase strand slippage in DNA replication or by recombination errors. Uncovering alleles in F₂ plants is the key to producing evolutionary varieties, as rice blast resistance traits are not only qualitative or quantitative but both together. These traits are called threshold traits, which are polygenic and expressed in categories and give two types of resistance: partial (quantitative) and complete (qualitative).

Table 9. Survey of the expected DNA fragments of the three primers in ten rice genotypes.

Genotypes/Fragments	Size (bp)	Giza 177	Giza 179	Giza 182	Sakha 101	Sakha 104	Sakha 106	GZ1368-5-5-4	GZ6296-12-1-2-1-1	Puebla	Hispagrán
YL155/YL87.1 (Pita2)	1040	0	0	0	0	0	0	0	0	1	0
RG64-431/RG64-432.1 (Pi2)	1450	1	1	1	0	1	0	1	0	0	1
RG64-431/RG64-432.2	1400	1	1	0	0	1	0	1	1	1	1
RG64-431/RG64-432.3	1000	1	1	0	0	1	0	1	0	0	1
RG64-431/RG64-432.4	850	1	1	0	0	1	0	1	0	0	1
RG64-431/RG64-432.5	600	1	1	1	0	1	0	1	0	0	1
RG64-431/RG64-432.6	350	1	1	1	0	0	0	1	0	0	0
RG64-431/RG64-432.7	250	1	1	0	0	0	0	1	0	0	1
RG64-431/RG64-432.8	130	1	1	0	0	0	0	1	0	0	1
RG64-431/RG64-432.9	100	1	1	0	0	0	0	1	0	0	0
Total		9	9	3	0	5	0	9	1	1	7
PBA12.1 (Pi9)	2000	0	0	0	0	0	0	0	1	0	0
PBA12.2	1550	1	1	1	1	0	1	1	1	0	0
PBA12.3	1500	1	1	1	1	1	1	1	1	1	1
PBA12.4	1400	1	1	1	1	1	1	1	1	1	1
PBA12.5	1000	1	1	1	1	1	0	0	0	0	0
PBA12.6	900	0	1	1	1	0	0	1	1	0	0
PBA12.7	800	1	0	0	1	0	1	0	0	0	0
PBA12.8	750	1	0	0	1	1	1	0	0	0	0
PBA12.9	700	0	1	1	0	0	0	1	1	1	1
PBA12.10	600	1	1	1	1	0	0	0	1	0	0
PBA12.11	550	1	1	1	1	1	1	0	1	1	1
PBA12.12	500	0	0	0	1	1	1	1	1	0	0
PBA12.13	400	0	1	0	0	0	1	0	1	0	0
PBA12.14	350	0	0	0	0	0	1	0	1	0	0
Total		8	9	8	10	6	9	6	11	4	4

Table 10. DNA fragments of the three primers in the F2 population Giza 177 × Puebla (F_{2a}) and Giza 177 × Hispagrán (F_{2b}).

Markers/F ₂ Plants	Size (bp)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
YL155/YL87.1	1040	F _{2a}	1	1	1	1	0	1	0	0	1	0	1	0	0	1	0	1	1	1
YL155/YL87.1	1040	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG64-431/RG64-432.1	1450	F _{2a}	1	1	1	0	1	1	1	1	0	0	0	1	0	1	0	0	1	1
RG64-431/RG64-432.2	1400	F _{2a}	1	1	1	1	1	0	1	1	0	0	1	1	0	1	0	1	1	1
RG64-431/RG64-432.3	1000	F _{2a}	1	1	1	0	1	0	1	1	0	0	1	1	0	1	0	1	1	1
RG64-431/RG64-432.4	850	F _{2a}	1	1	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	1
RG64-431/RG64-432.5	600	F _{2a}	1	1	1	0	0	0	0	1	0	0	1	1	0	1	0	1	1	1
RG64-431/RG64-432.6	350	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
RG64-431/RG64-432.7	250	F _{2a}	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
RG64-431/RG64-432.8	130	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG64-431/RG64-432.9	100	F _{2a}	1	1	1	1	1	0	1	1	1	0	0	1	0	1	1	1	1	1
Total			7	7	7	4	5	1	5	6	1	0	4	6	0	6	1	5	7	8

Table 10. Cont.

Markers/F ₂ Plants	Size (bp)		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
RG64-431/RG64-432.1	1450	F _{2b}	0	1	1	1	1	0	1	1	0	0	0	0	0	1	0	1	1	1
RG64-431/RG64-432.2	1400	F _{2b}	0	1	1	1	1	0	1	1	0	0	1	1	1	0	1	1	1	1
RG64-431/RG64-432.3	1000	F _{2b}	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	1
RG64-431/RG64-432.4	850	F _{2b}	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0
RG64-431/RG64-432.5	600	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG64-431/RG64-432.6	350	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG64-431/RG64-432.7	250	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RG64-431/RG64-432.8	130	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0
RG64-431/RG64-432.9	100	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total			0	2	2	2	2	0	2	2	0	0	3	3	3	3	4	5	2	3
PBA12.1 (Pi9)	2000	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.2	1550	F _{2a}	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1
PBA12.3	1500	F _{2a}	1	0	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1
PBA12.4	1400	F _{2a}	1	1	0	1	1	0	0	0	0	0	1	0	1	0	1	1	0	1
PBA12.5	1000	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.6	900	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.7	800	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.8	750	F _{2a}	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.9	700	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.10	600	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.11	550	F _{2a}	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
PBA12.12	500	F _{2a}	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
PBA12.13	400	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PBA12.14	350	F _{2a}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total			3	2	1	3	3	1	0	0	1	1	5	1	3	1	3	4	1	4
PBA12.1 (Pi9)	2000	F _{2b}	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
PBA12.2	1550	F _{2b}	0	0	1	0	0	1	1	0	0	1	1	0	0	1	0	0	0	0
PBA12.3	1500	F _{2b}	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	0	0	0
PBA12.4	1400	F _{2b}	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
PBA12.5	1000	F _{2b}	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
PBA12.6	900	F _{2b}	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1
PBA12.7	800	F _{2b}	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PBA12.8	750	F _{2b}	1	0	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0
PBA12.9	700	F _{2b}	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PBA12.10	600	F _{2b}	0	0	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	1
PBA12.11	550	F _{2b}	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PBA12.12	500	F _{2b}	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1
PBA12.13	400	F _{2b}	0	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
PBA12.14	350	F _{2b}	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total			3	0	11	8	6	12	13	4	5	7	8	6	6	7	6	6	6	6

4. Conclusions

Our findings suggest that aggregating all alleles of blast broad-spectrum resistant genes is difficult. The detection of fragments associated with the gene using molecular markers to clarify its presence and absence in plants is an important tool to help in the selection of second-generation plants to save time and effort, in addition to choosing parents in hybridization programs. The use of co-dominant molecular markers is important to elucidate the fragments related to the presence of the gene, and then to clarify the presence of re-combinations to form a new allele that may be effective in resistance in the offspring. Also, not using molecular scanning of parents before hybridization for a studied trait and confirming the transmission of the trait to the second generation after phenotypic selection is considered blind selection and depends on throwing dice.

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