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Full Length Research Paper

Identification of leaf rust resistant genes *Lr9, Lr25, Lr28, Lr29* and *Lr67* in ten Egyptian wheat cultivars using molecular markers

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Breeding for resistance is an efficient strategy to manage wheat leaf rust (Lr) caused by *Puccinia triticina* f. sp. *tritici*. However, a prerequisite for the directed use of Lr genes in breeding and detection of new races virulent is a detailed knowledge on these genes present in wheat cultivars. Molecular markers are ideal for the identification of resistance genes in wheat genotypes with unknown genetic background. Therefore, molecular markers were conducted using specific SSR primers to screen ten out of fifteen Egyptian wheat cultivars which exhibited high resistance against *P. triticina* f. sp. *tritici* in four locations (Dakahlia, Kafr el-Sheikh, Beheira and Sharqia) during seasons 2010/2011 and 2011/2012. The obtained results showed that *Lr9* was present in two cultivars namely Sids-12 and Sids-13, while *Lr25* was found in all ten tested cultivars except Gemmeiza-11 cultivar. *Lr28* was found in five cultivars, that is, Giza-168, Sids-12, Misr-2, Sakha-94 and Misr-1. On the other hand, *Lr25*, *Lr29* and *Lr67* genes were detected in all tested cultivars. Thus, the uses of molecular markers facilitate the incorporation of the major leaf rust resistance genes (Lr genes) responsible for resistance into new cultivars and the pyramiding of these genes. Further suggestion shows that the amplification of specific PCR products is an easy and repeatable method, which will be useful in automating the detection of resistance genes in released wheat breeding lines.

Key words: Wheat, leaf rust, *Puccinia triticina*, wheat cultivars, resistance genes, molecular markers.

INTRODUCTION

Wheat plays a central role in Egypt's food economy, both in terms of production and consumption. Gap in production and consumption is escalating due to the ever-increasing population. Wheat production is also decreasing due to the attack of certain diseases like rusts, smuts, powdery mildews, etc. Rust diseases of wheat are among the oldest plant diseases known to man. Leaf rust is the most destructive and devastating disease due to its time of appearance, nature of attack, regular occurrence and prolonged growing season that is prevalent for its development in the wheat growing areas of the world (Ahmad et al., 2010). Since the discovery of rust, numerous studies have been conducted on the life cycles of rust pathogens and their management. Due to airborne nature of the disease, use of chemicals is neither economical nor feasible on a large scale. The only economic and practical control of rust diseases can be achieved through genetic resistance (Boulot, 2007; Samsampour et al., 2010). The most environmentally sound, low cost method of controlling leaf rust is to breed and grow resistant wheat varieties. So far, over 60 leaf rust resistance genes (Lr genes) have been identified and localized on wheat chromosomes (El-Shamy and Mousa, 2004). In addition, a number of temporarily designated resistance genes and quantitative trait loci (QTLs) are able to provide total or partial protection against various rust pathotypes (Hiebert et al., 2010). The effectiveness of resistance genes depends on the composition of the pathogen population. As this changes dynamically, new pathotypes virulent to the given resistance gene multiply

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from time to time, so the resistance of a variety is not a constant trait. Any variety carrying a single resistance gene may become susceptible within a short time. The postulation of resistance genes is traditionally carried out using rust isolates with known virulence (Khan et al., 1997), but this procedure is extremely time-, space- and labour-intensive and cannot be employed if no differential fungal isolate is available. In many cases, resistance genes can only be identified using molecular markers (Knott, 1989). Over the last 15 years, many efficient markers for leaf rust resistance genes have been described. Accordingly, molecular markers are used for two purposes in resistance breeding: (1) to identify resistance genes in varieties and lines where the genetic background is unknown (i.e, gene detection) (Kolmer et al., 2007) (2) to monitor the incorporation of designated resistance genes or QTLs into elite wheat genotypes, i.e, MAS (marker-assisted selection). Microsatellites (simple sequence repeats (SSRs)) are repeating sequences of 2-5 base pairs of DNA. SSRs are typically co-dominant and used for gene detecting, gene duplication or deletion, MAS and fingerprinting. Thus, we used the SSR markers in this study to identify some leaf rust resistance genes in selected ten Egyptian bread wheat cultivars.

MATERIALS AND METHODS

Evaluation of 15 Egyptian wheat cultivars and four monogenic lines under field condition

A total of 15 wheat cultivars: Sakha-61, Sakha-69, Sakha-93, Sakha-94, Sakha-95, Gemmeiza-7, Gemmeiza-9, Gemmeiza-10, Gemmeiza-11, Sids-1, Sids-12, Sids-13, Giza-168, Misr-1 and Misr-2, and four resistance monogenic lines (Lr genes) *Lr9, Lr25, Lr28,* and *Lr29* were evaluated under field condition at four locations namely Dakahlia, Kafr el-Sheikh, Beheira and Sharqia during two seasons 2010/2011 and 2011/2012 for leaf rust resistance (Table 1). These cultivars were sown in 3 m long rows, with 30 cm apart and 5 g seed rate for each row. The experiment was surrounded by 1.5 m belt of highly susceptible varieties, that is, *Morocco and Triticum spleta saharences*, served as a spreader for leaf rust infection. These spreaders were artificially inoculated using a mixture of races in addition to the natural infection during late tillering and early booting. Rust reaction was expressed in five types: immune = (0), resistant = (R), moderately resistant = (MR), moderately susceptible = (MS) and susceptible = (S) (Stakman et al., 1962). Then rust reaction was transformed to average coefficient of infection (ACI) values according to the methods adopted by Saari and Wilcoxson (1974).

Plant material

Ten out of fifteen Egyptian wheat cultivars: Sakha-94, Sakha-95, Gemmeiza-9, Gemmeiza-10, Gemmeiza-11, Sids-12, Sids-13, Giza-168, Misr-1 and Misr-2 and five resistance monogenic lines *Lr9, Lr25, Lr28, Lr29 and Lr67* were chosen as plant materials for detection of leaf rust resistance genes using molecular markers.

Molecular markers

The specific SSR primers used to verify the presence of *Lr9, Lr25, Lr28, Lr29* and *Lr67* genes in 10 cultivars are listed in Table 2. This part of the investigation was carried out at the Molecular Biology Laboratory, Faculty of Agriculture Research Park (FARP), Faculty of Agriculture, Cairo University. Giza, Egypt.

DNA extraction

A modified method based on the protocol of Dellaporta et al., 1983 was conducted for extraction of total genomic DNA.

PCR amplification

Polymerase chain reaction was performed in thermocycler (Rocorbett-Research, CG1-96) in 25 µL reaction volume containing: 2.5 µL 50 ng/µL of genomic DNA, l µL of each primer (10 pmol, F and R) and 8 µL MQ H2O (Devos and Gale, 1992). Amplification products were electrophoresed at 100V/1h. After electrophoresis, the gel was stained with ethidium bromide and bands were visualized using UV light and photographed with a Syngen UV visualizer (gel documentation system, G:BOX). The Mid-Range DNA Ladder 100bp-3kbp linear scale (Jena Bioscience) was used as standard marker for molecular weight.

RESULTS

Evaluation of 15 Egyptian wheat cultivars and four monogenic lines under field condition

Fifteen wheat cultivars and four monogenic lines (Lr genes) were evaluated against leaf rust under field condition in four locations: Kafr el-Sheikh, Beheira, Dakahlia and Sharqia during growing seasons 2010/2011 and 2011/2012. Data presented in Table 1 revealed that the wheat cultivars Giza-168, Misr-1, Sakha-94, Misr-2, Sakha-95, Sids-13, Sids-12, Gemmeiza-9, Gemmeiza-10 and Gemmeiza-11 showed high resistance where the rust severity mean values were 0.47, 0.72, 1.00, 1.05, 1.15, 2.25, 3.75, 6.75, 8.50 and 9.75% respectively during the two seasons. On the other hand, the considered highly susceptible wheat cultivars were Gemmeiza-7, Sids-1, Sakha-61, Sakha-93 and Sakha-69 and showed high levels of rust severity, i.e, 77.50, 71.25, 60.00, 60.00 and 51.25% respectively.

Table 1. Leaf rust severity on 15 wheat cultivars and 4 monogenic lines in 4 locations during seasons 2010/2011 and 2011/2012.

Cultivar	Rust severity (2010/2011)				Rust severity (2011/2012)				Mean (ACI*)
	Kaffr el-Sheikh	Beheira	Dakahlia	Sharqia	Kaffr el-Sheikh	Beheira	Dakahlia	Sharqia	
Sakha-61	60S	70S	80S	60S	50S	30S	60S	70S	60
Sakha-69	40S	80S	40S	40S	70S	60S	20S	60S	51.25
Sakha-93	40S	80S	60S	80S	60S	50S	40S	70S	60
Sakha-94	0	5R	5MR	5R	0	0	5MS	0	1
Sakha-95	5R	5MR	0	5R	5R	5MR	5R	TrMR	1.15
Gemm.-7	70S	90S	90S	100S	70S	80S	40S	80S	77.5
Gemm.-9	5S	20MS	5MS	10S	5MR	10S	5MR	5S	6.75
Gemm.-10	30MS	10MS	5S	5S	10MR	10S	10S	5MR	8.5
Gemm.-11	20MS	10S	20MS	10S	10MR	10S	10S	5MR	9.75
Giza-168	TrR	0	TrMR	0	0	0	10R	0	0.47
Sids-1	80S	90S	90S	90S	60S	70S	50S	40S	71.25
Sids-12	5MR	TrMS	5MS	5MR	TrMR	0	TrMS	20MS	3.75
Sids-13	10MS	0	5MS	0	5MR	0	10MR	0	2.25
Misr-1	0	TrMR	TrMR	0	0	TrMS	0	5R	0.72
Misr-2	0	5MR	5MR	0	0	TrMS	0	5MR	1.05
Lr 9	0	10R	0	0	10R	TrR	5R	10R	0.95
Lr25	0	0	0	0	0	0	0	0	0
Lr 28	5MS	0	0	TrR	0	0	10MR	0	1.07
Lr29	5MR	TrMS	0	10MR	10MS	5MS	10MS	0	3.55

*ACI = Average coefficient of infection.

Table 2. Primer names, sequences, PCR annealing temperature and references for Lr gene associated markers used in this study.

S/N	Gene	Name	Primer sequences (5'-3')	Annealing temperature	References
1	*Lr9*	J 13/1 J 13/2	TCC TTT TAT TCC GCA CGC CGG CCA CAC TAC CCC AAA GAG ACG	62°C	Schachermayr et al. (1994)
2	*Lr25*	Lr25F20 Lr25R19	CCA CCC AGA GTA TAC CAG AG CCA CCC AGA GCT CAT AGA A	57°C	Urbanovich et al. (2006)
3	*Lr28*	*Lr 28-01* *Lr 28-02*	CCC GGC ATA AGT CTA TGG TT CAA TGA ATG AGA TAC GTG AA	50°C	Vanzetti et al. (2011)
4	*Lr29*	Lr29F24 Lr29R24	GTG ACC TCA GGC AAT GCA CAC AGT GTG ACC TCA GAA CCG ATG TCC ATC	65°C	Urbanovich et al. (2006)
5	*Lr67*	F R	GTG ACC TCA GAA CCG ATG TCC ATC GCA AGG AAG AGT GTT CAG CC	59°C	Vida et al. (2009)

Likewise, the monogenic line *Lr25* showed highly resistance (0 Disease Severity) to leaf rust disease in four locations during the two seasons followed by *Lr9* (0.95%), *Lr28* (1.07%) and *Lr29* (3.55%) (Table 1).

Molecular markers

Data in Table 3 revealed the resistance genes detected in the selected wheat cultivars using specific SSR primers. The polymorphic survey revealed that out of the 10 cultivars, the marker for *Lr9* was identified as a fragment of 300 bp in two cultivars namely: Sids-12 and Sids-13, while eight cultivars: Sakha-94, Sakha-95, Gemmeiza-9, Gemmeiza-10, Gemmeiza-11, Giza-168, Misr-1 and Misr-2 did not show the presence of *Lr9* (Figure 1).

Likewise, the marker for *Lr25* was identified as a fragment of 250 bp in nine cultivars: Sakha-94, Sakha-95, Gemmeiza-9, Gemmeiza-10, Sids-12, Sids-13,

Table 3. Lr genes detected with PCR based markers in ten Egyptian wheat cultivars.

S/N	Cultivar	Lr gene				
		Lr9	***Lr25***	***Lr28***	***Lr29***	***Lr67***
1	Sakha 94	-	+	+	+	+
2	Sakha 95	-	+	-	+	+
3	Gemmeiza 9	-	+	-	+	+
4	Gemmeiza 10	-	+	-	+	+
5	Gemmeiza 11	-	-	-	+	+
6	Sids 12	+	+	+	+	+
7	Sids 13	+	+	-	+	+
8	Giza 168	-	+	+	+	+
9	Misr 1	-	+	+	+	+
10	Misr 2	-	+	+	+	+

(+) = presence of Lr gene in wheat cultivars and (-) = absence of Lr gene in wheat cultivars.

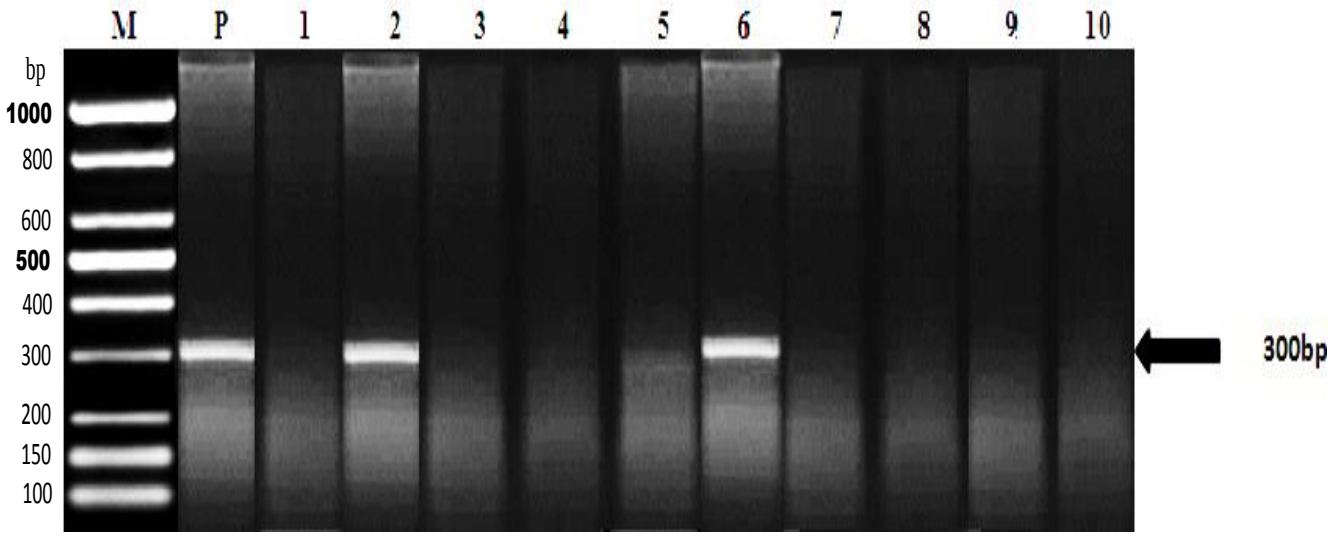

Figure 1. Electrophoretic amplified pattern of DNA extracted from 10 cultivars using the specific primers of *Lr9*. M = Mid-Range DNA Ladder, P = positive, Lane 1 = Giza-168, Lane 2 = Sids-12, Lane 3 = Misr-2, Lane 4 = Sakha-95, Lane 5 = Sakha-94, Lane 6 = Sids-13, Lane 7 = Gemmeiza-10, Lane 8 = Gemmeiza-9, Lane 9 = Misr-1 and Lane 10 = Gemmeiza-11.

Giza-168, Misr-1 and Misr-2 (Figure 2).

The marker for resistance gene *Lr28* was found in five cultivars, i.e, Giza-168, Sids-12, Misr-2, Sakha-94 and Misr-1 but was absent in the remaining cultivars (Figure 3). In contrast, markers for *Lr*29 and *Lr*67 were identified in all tested cultivars which would indicate that these cultivars possess these two genes (Figures 4 and 5 respectively).

DISCUSSION

An important field in which molecular markers are used in wheat breeding is the determination of designated resistance genes in genotypes where the genetic background has not yet been clarified, like most commercial cultivars. Molecular markers can be used for several different applications (McIntosh, 1988) including: germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenic analysis, etc.

As regard to the performance of certain Egyptian wheat cultivars under field conditions, the evaluation of 15 cultivars indicated that the vast majority of cultivars exhibited high resistance with the exception of cultivars Sakha-61, Sakha-69, Sakha-93, Gemmeiza-7 and Sids-1. Similar results were recorded by Mcintosh et al. (2008), Melchinger (1990), Naik et al. (1998), and Pathan and Park (2006) who confirmed that the rust severity of wheat cultivars Giza 168, Sakha 94, Gemmeiza 9 and Gemmeiza 10 were low compared to susceptible

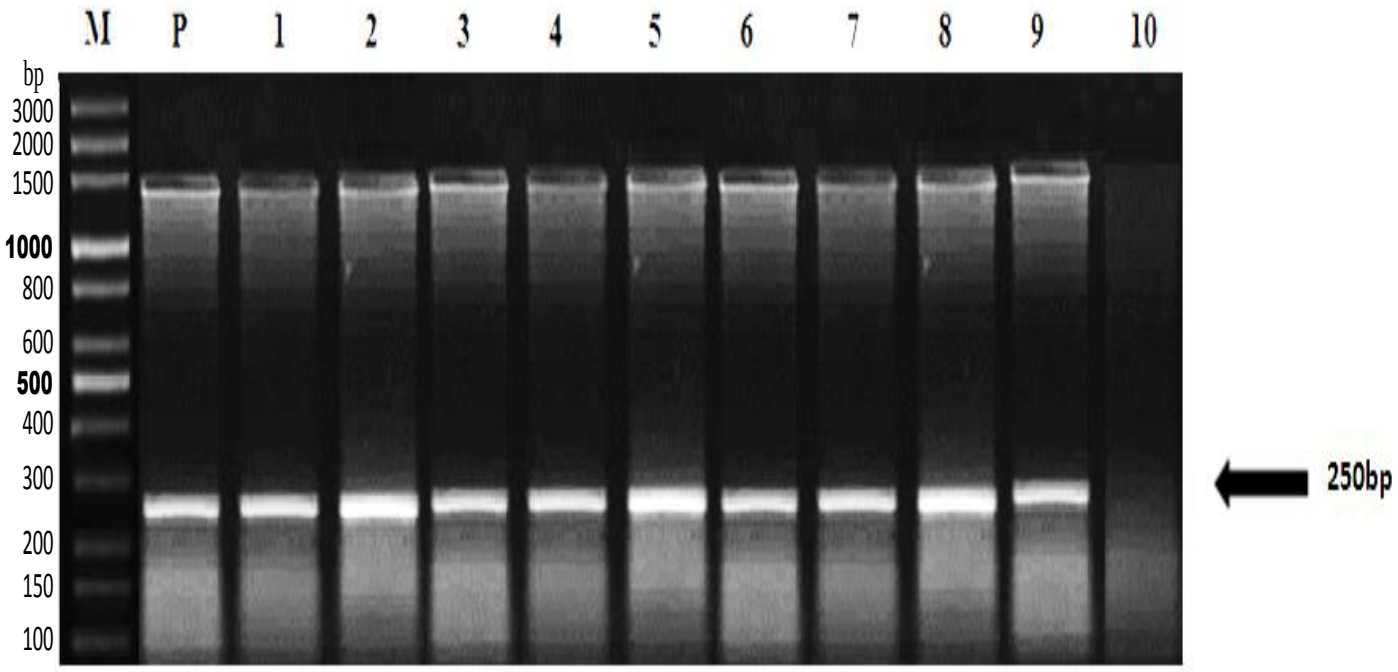

Figure 2. Electrophoretic amplified pattern of the specific primers for *Lr25*. M = Mid-Range DNA Ladder, P = positive, Lane 1 = Giza-168, Lane 2 = Sids-12, Lane 3 = Misr-2, Lane 4 = Sakha-95, Lane 5 = Sakha-94, Lane 6 = Sids-13, Lane 7 = Gemmeiza-10, Lane 8 = Gemmeiza-9, Lane 9 = Misr-1 and Lane 10 = Gemmeiza-11.

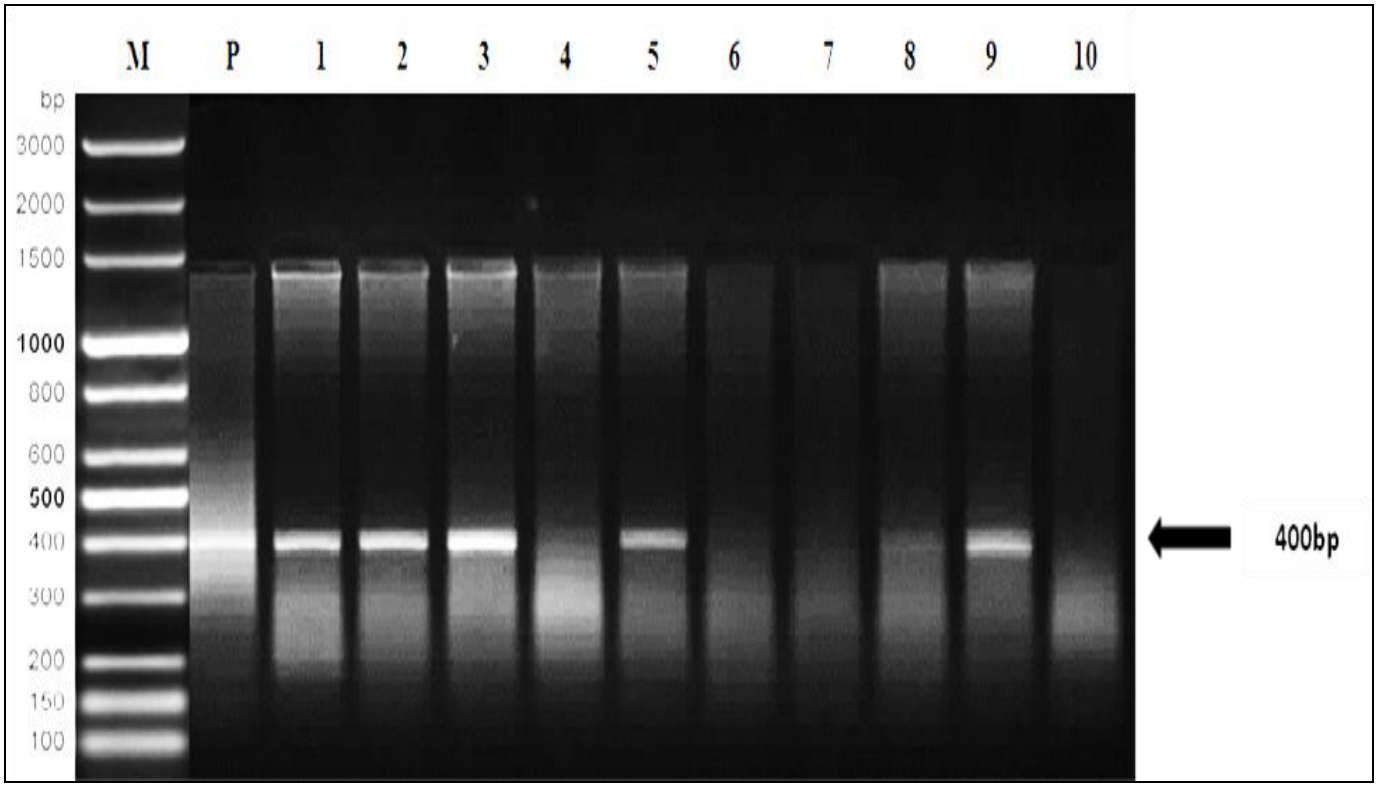

Figure 3. Electrophoretic pattern of the specific primers for *Lr28*. M= Mid-Range DNA Ladder, P=positive, Lane 1= Giza-168, Lane 2= Sids-12, Lane 3= Misr-2, Lane 4= Sakha-95, Lane 5= Sakha-94, Lane 6= Sids-13, Lane 7= Gemmeiza-10, Lane 8= Gemmeiza-9, Lane 9= Misr-1 and Lane 10= Gemmeiza-11.

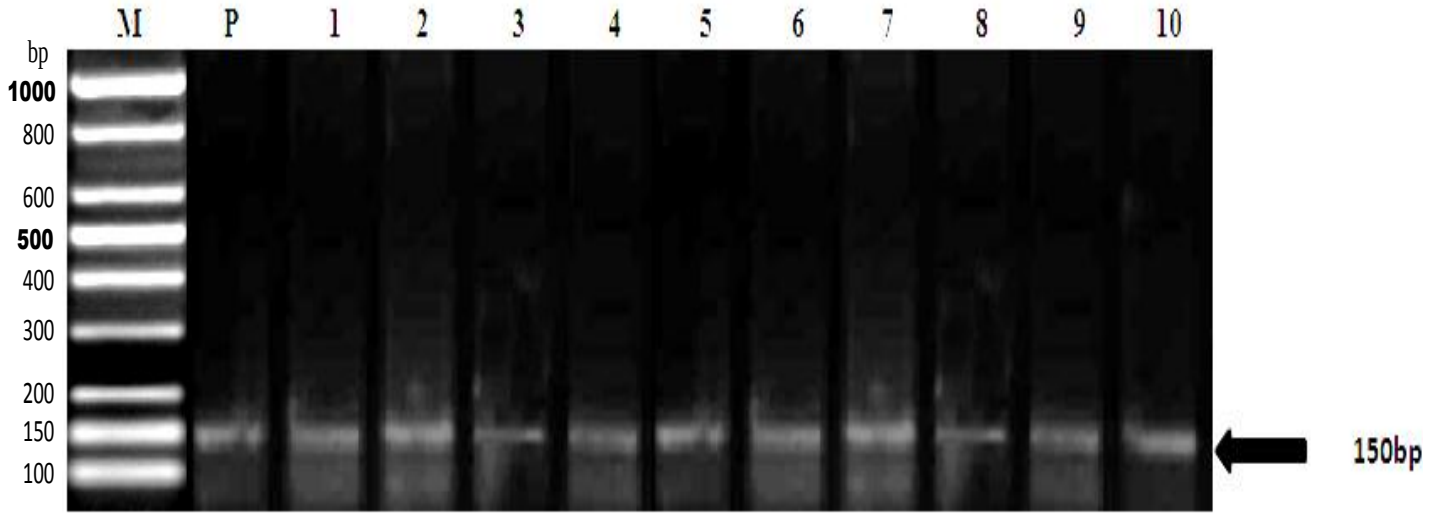

Figure 4. The Electrophoretic amplified pattern of the specific primers for *Lr29*. M = Mid-Range DNA Ladder, P =positive, Lane 1 = Giza-168, Lane 2 = Sids-12, Lane 3 = Misr-2, Lane 4 = Sakha-95, Lane 5 = Sakha-94, Lane 6 = Sids-13, Lane 7 = Gemmeiza-10, Lane 8 = Gemmeiza-9, Lane 9 = Misr-1 and Lane 10 = Gemmeiza-11.

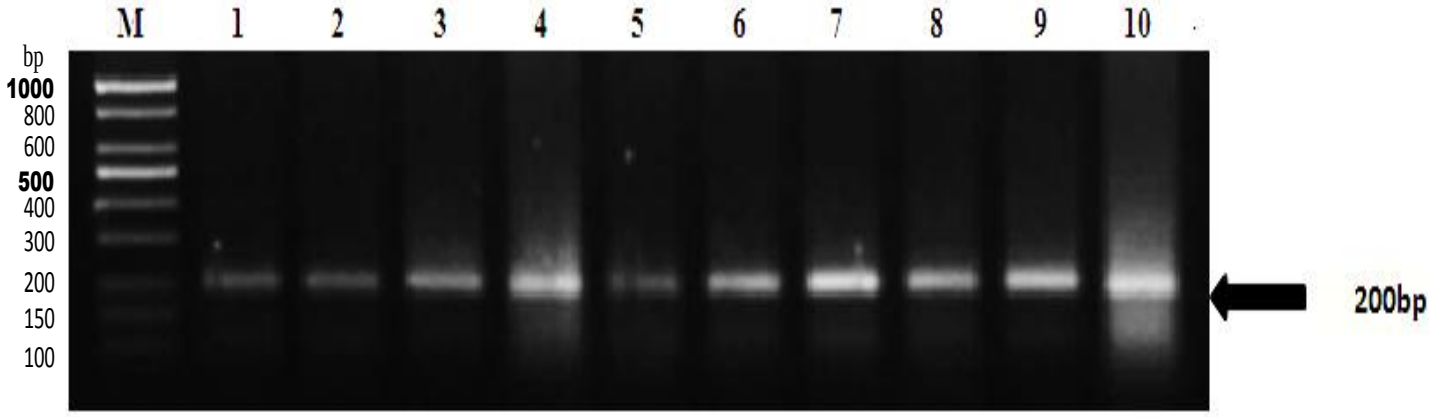

Figure 5. Electrophoretic amplified pattern of 10 cultivars DNA using the specific primers of *Lr67*. M = Mid-Range DNA Ladder, P = positive, Lane 1 = Giza-168, Lane 2 = Sids-12, Lane 3 = Misr-2, Lane 4 = Sakha-95, Lane 5 = Sakha-94, Lane 6 = Sids-13, Lane 7 = Gemmeiza-10, Lane 8 = Gemmeiza-9, Lane 9 = Misr-1 and Lane 10 = Gemmeiza-11.

cultivars. He also showed that cultivars Sids 1, Giza 139 and Giza 160 exhibited the highest rust severity during 2006/2007 and 2007/2008 growing seasons.

Likewise, we concluded that *Lr9, Lr25, Lr28, Lr29* and *Lr67* provide a good degree of resistance. So these genes should be taken in consideration in breeding programs for successful rust resistance. However, one of the main disadvantages in using single gene resistance is rapid changes in predominant rust pathogen races (pathotypes) in nature; single-gene resistance in a cultivar may become ineffective soon after it is released. For example, in USA, *Lr9* was initially used in soft red winter wheat in the 1970s and initially gave complete immunity to leaf rust. In spite of this, within a few years, races with virulence to *Lr9* appeared and soon became widespread in the Easter USA (Procunier et al., 1995). Therefore, identification and introgression of resistance genes into elite cultivars became an essential way for wheat breeding programs for resistance.

Ten resistance Egyptian wheat cultivars out of 15 were selected for molecular markers identification and the results obtained proved that the resistance was due to the presence of resistance genes, i.e, *Lr9*, *Lr25, Lr28, Lr29*, and *Lr67*. The gene *Lr9* was detected only in two cultivars Sids-12 and Sids-13. This gene has the wide range of effectiveness as a useful source of resistance when deployed in a combination with complementary *Lr* genes like *Lr51*, *Lr21*, etc. *Lr9,* derived from *T.*

umbellulata, has also been detected in low frequency in some European countries and in the USA (Putnik-Deliã, 2008; Rafalski et al., 1996).

The gene *Lr28* was detected in five cultivars namely Giza-168, Sids-12, Misr-2, Sakha-94 and Misr-1. They also carried *Lr25, Lr29* and *Lr67* genes that could explain the high resistance detected in these cultivars. Therefore, we recommend the use of these cultivars as a parental in leaf rust resistance breeding programs for gene pyramiding where the cultivar carries more than one gene in which it could be planted in many locations in Egypt and other counters. Still, a rigorous evaluation of the agronomic effect of new resistance gene combinations on a host phenotype will be required to discard an eventual decrease in host fitness.

Conclusion

Genetic resistance is the most economic and effective means of reducing yield losses to this disease. However, breeding disease resistance genotypes is a continuous process, and plant breeders need to add new effective genes to their breeding materials. Knowledge of the identity of the leaf rust resistance genes in released cultivars is essential for the incorporation of the resistance genes into breeding programs and maintenance of a diversity of resistance in commonly grown cultivars. We identified in this work *Lr9, Lr25, Lr28, Lr29* and *Lr67* in ten resistance Egyptian wheat cultivars in which they could use in building wheat breeding program.

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