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Identification of Isozyme and Molecular Markers Linked To Leaf Rust Resistance in New Egyptian Wheat lines

Ramadan M. Esmail[1], Abdel Sattar A. Abdel Sattar[1], Mohamed A. Abou-Ellail[2], Heba A. Mahfouze[1], Hoda M.A. Waziri[3], Sherin A. Mahfouze[1*]

1. National Research Centre, Genetic Engineering and Biotechnology Division, Genetics and Cytology Department, Dokki, 12622, Egypt.
2. University of Aswan, Faculty of Agriculture and Natural Resources, Department of Genetics, Aswan, Egypt.
3. Virus and Phytoplasma Department, Plant Pathology Research Institute, Agriculture Research Center, Giza, Egypt.
Corresponding author email: sherinmahfouze@yahoo.com

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A B S T R A C T

Leaf rust is a crucial disease of wheat (Triticum aestivium L.) in Egypt and worldwide. It causes by fungi Puccinia triticina. Leaf rust could be controlled by genetic resistance to limit yield damages. In this study, we evaluate new wheat lines for resistance to leaf rust disease. In addition, determine the activities of polyphenol oxidase and peroxidase in leaf rust resistance wheat lines compared with the healthy control. Also, we identify Inter-simple sequence repeat (ISSR-PCR) markers linked to Lr resistant genes at the seedling stage. All wheat lines were resistant to leaf rust at seedling stage. However, under the field conditions three lines No. 3, 7 and 8 were resistant, while other seven were susceptible. Consequently, it could be concluded that lines No. 3, 7 and 8 were resistant to leaf rust in both stages. Eight anchored ISSR primers were screened to identify polymorphic bands between the leaf rust resistance and control wheat plants. A total of 104 scorable fragments were amplified of which 54 monomorphic and 46 bands were polymorphic resulting in a polymorphism of 44.23%. The largest number of ISSR-PCR specific markers scored in leaf rust resistance wheat line No. 10 (three markers), followed by leaf rust resistance wheat lines No. 2, 3, 4 and 8 (two). The lowest number of specific bands existed in resistance line No. 1 (one unique marker). These markers could be applied in wheat breeding programs, as marker assisted selection. By a backcross program was achieved by recurrent parents between the susceptible high-quality wheat and the resistant gene carrying ones as donor parents.

Key words: *Triticum aestivum L, Puccinia triticina, peroxidase, polyphenol oxidase, ISSR-PCR*

Introduction

Leaf rust is a crucial disease of wheat (Triticum aestivium L.) in Egypt and worldwide. It causes by Puccinia triticina fungi (Germán et al. 2007; Kolmer et al. 2007; Gennaro et al. 2009; Imbaby et al. 2014). Abdel Hak et al . (1980) determined leaf rust infection damages in Egypt up to 50%. The gene-for-gene interaction between P. triticina and its host is appeared by the incompatible interaction between a rust isolate carrying the avirulence gene and a wheat plant contained on the resistance gene (Lr). P. triticina fungi play a main role in leaf rust epidemiology (Kolmer 2005). Leaf rust (Lr) resistance gene(s) have been applied successfully in breeding programs in order to develop leaf rust resistance new wheat lines. However, single R-genes tend to be quickly overcome by changes in the P. triticina strains. More durable resistance could be applied by the gene pyramiding i.e., the stacking of multiple Lr genes, such as the Canadian cultivar 'Pasqua' (Kolmer 2001; Liu and Kolmer 1998). More than 50 major leaf rust resistance (Lr) genes have been identified McIntosh et al. (2007), approximately half of which originate from both wild and cultivated wheat. Lind and Gultyaeva (2007); Hanzalová et al. (2008) showed that Lr19 to be one of the most effective across time and space. Wheat cultivars carrying leaf rust resistance genes can avoid yield damages by developing near isogenic lines (NILs) carrying the resistance gene to leaf rust. For Lr genes in wheat, Thatcher based NILs are available. The traditional method of transferring one or more resistance genes to a single wheat cultivar depend on the field and greenhouse selection with different races, which is very laborious and time depleting.

Recently, DNA-based markers have shown great promise in the time saving and cost for determining resistance genes (Purnhauseri et al. 2000). Inter-simple sequence repeat (ISSR) fingerprinting requires a PCR amplification of DNA using primers based on a repeat sequence anchored at the 5' or 3' end by one to three

arbitrary nucleotides (Zietkiewicz et al. 1994). It is a useful technique, highly reproducible and requires a small quantity of DNA template, allowing an early selection of the progeny. PCR products can be separated on agarose gel and the results are available within hours (Fang and Roose 1997).

In this study, we evaluate new wheat lines originated from a final wheat breeding program for resistance to leaf rust. Also, identify molecular marker (ISSR-PCR) linked to the resistance loci from these lines that can be used as marker-assisted selection in breeding programs.

Materials And Methods

Leaf rust inoculation

At seedling stage

Ten wheat lines (20 plants from each line) were evaluated to leaf rust pathotypes under the greenhouse conditions at Wheat Disease Department, Plant Pathology Institute, Agricultural Research Center (Table 1). The tested lines were sown (10 cm) diameter plastic pots filled with peat moss and vermiculite in the greenhouse. Seven days old wheat seedlings were artificially inoculated with a mixture of leaf rust races i.e. TSTTK, PJTLS, NKTST and PTTST during the season 2012/13. Inoculated seedlings were placed for 24 h in a dark, humid chamber at 19°C and then moved to the glasshouse, under a 16-h photoperiod and a 25°C (daylight) and 22°C (night) temperature regime. After 12 days of inoculation, the infection type descriptions still in use are based on the original scales proposed by Stakman et al. (1962) to leaf rust. The rust reactions 0, 0;, 1, and 2 were considered resistant (R) response, while 3 and 4 were considered susceptible (S) response.

At adult stage

This experiment was carried out under the field conditions at Nubariya Agric. Res. Station for one season i.e. 2012/13. The ten tested wheat lines (Table 1) were planted in three replicates with 2 m length single row, each 30 cm apart. The experiment was surrounded by spreader rows planted with a mixture of the highly susceptible varieties i.e., 'Morocco', 'Thatcher' and Triticum spelta cv 'Saharinsis'. Randomization was not used in planting these lines, since it seemed to be unnecessary Broers (1987) because of the high proportion of the infection reaching the tested genotypes from the spreader rows. Seventy-five days old wheat lines (prebooting stage) were artificially inoculated with a mixture of uredospores of the prevalent races (TSTTK, PJTLS, NKTST and PTTST) mixed with talcum powder at a rate of 1 (spores): 25 (talcum powder) (v:v) according to the method described by Tarvet and Cassell, (1951). The rust response was recorded after the heading stage by combining severity from 0 to 100% (percent of infection) according to the modified Cobb scale (Peterson et al. 1948) and reaction (type of reaction) (Johnston and Mains 1932).

Isozymes electrophoresis

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations between leaf rust resistance wheat lines and the healthy control at the seedling stage using polyphenol oxidase (PPO) and peroxidase (POD) isozymes according to Stegemann et al. (1985). 500 mg fresh leaves were homogenized in liquid N_2 and 100 µl of 0.2 M Phosphate buffer was added (pH 7.0) was adjusted by potassium phosphate, monobasic) and 10 µl of 2-mercaptoethanol before centrifugation at 14000 rpm for 15 min at 4°C. The supernatant was stored at a temperature of -20°C until isozyme analysis. Polyphenol oxidase isozymes were detected according to Baaziz et al. (1994), in which the gel was immersed in a solution containing 0.1% 1-dihydroxyphenyl alanine solubilized in 0.05 M phosphate buffer pH 7.5. For peroxidase, benzidine-dihydrochloride HCl of 0.125 gm and 2 ml glacial acetic acid and was completed with dsH_2O up to 50 ml. Gel was placed into this solution and 5 drops of hydrogen peroxide was added. The gel was incubated at room temperature until bands appear (Brown 1978). Relative band mobility was measured in relation to the dye front and indicated by Rf values.

DNA isolation, ISSR-PCR conditions and gel electrophoresis

Genomic DNAs were extracted from leaves of ten wheat lines resistance and the healthy control according to Cetyl trimethylammonium bromide (CTAB) method (Silva and Procunier 1994). A total of eight anchored ISSR primers (Table 4) were used to amplify DNA (Life Technologies, Gaithersburg, Md.). Each 25-µl amplification reaction consisted of 2.5 µl 10X PCR buffer, 2.5 µl 25 mM $MgCl_2$; 0.5 µl 40 mM dNTPs; 1 µl Taq DNA polymerase (1 unit/µl); 2 µl 0.4 µM primer. Amplification was carried out in DNA thermocycler (Biometra, Germany) under the following conditions: one cycle of 3 min at 94°C, followed by 28 cycles for 45 sec at 94°C, 30 sec at 52°C and 2 min at 72°C; a final extension for 6 min at 72°C. Amplification products were separated on a 1% agarose gel containing 1X TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) and 0.5 µg/ml ethidium bromide at 90 V. The Gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

Results And Discussion

Evaluation of wheat lines against infection with leaf rust under the greenhouse and the field conditions

Resistance of the ten wheat lines to leaf rust disease at seedling (Figure 1) and adult plant stages are recorded in Tables (1 and 2). Some lines displayed resistance in both stages such as lines No. 3, 7 and 8. Nevertheless, the remaining

lines were resistant at the seedling stage and susceptible during the adult plant as lines No. 1, 2, 4, 5, 6, 9 and 10 (Tables 1 and 2). These results were in an agreement with Kharouf et al. (2010) who found that the cultivar Jpateco73S is susceptible during seedling and adult stages due to deficiency in resistance genes. In addition, Sadari cultivar was resistance during both stages. Therefore, the resistance gene during seedling stage gave the plant resistance at the adult stage. The Oxely APR cultivar was susceptible at the seedling stage but resistance during the adult stage. On the contrary, Avocet Yr 18 cultivar was susceptible only at the adult stage. This condition is due to the temperature effect. Mains and Jackson (1926) found that variance in cereal rusts has been evaluated by races on the host that differ in resistance. Most resistance genes were efficient at seedling stage and remain functional at the adult plant stage. Lr1, Lr10, and Lr21 genes were efficient during seedling and adult stages (Dyck and Kerber 1985). These genes gave low infection of hypersensitive flecks or uredinia encompassed by necrosis or chlorosis to a virulent rust strains. Leaf rust (Lr) resistance genes have conserved motifs that code for leucine repeat rich (LRR) and nucleotide binding site (NBS) proteins (Feuillet et al. 2003). These genes were identified in wheat T. aestivum (Lr1, Lr2a, Lr3, Lr10, Lr11) and related species such as Aegilops elongatum (Lr24), T. tauschii (Lr21), A. umbellulata (Lr9) and common rye, Secale cereale (Lr26) (Browder 1980). Race specific resistance genes have been identified that are strongly expressed during the adult plant stage, but weakly expressed at the seedling stage as Lr12 and Lr13, while others such Lr22a and Lr37 were derived from T. tauschiiand, A. ventricosa, respectively. For example, the seedling resistance genes, races with virulence to these adult plant resistance genes have eroded their efficiency. The other genes express a partial type of resistance that was demonstrated by fewer uredinia of variable size that were encompassed by chlorosis (Caldwell 1968). This type of resistance was expressed during adult stage, but at seedling stage could be susceptible. A key distinctive of these genes was that they give resistance to all P. triticina races, with no showed race specificity, although these genes individually did not give resistance that was appeared by a hypersensitive reaction without any uredinia developed. These genes have allowed long-term durable resistance because virulent forms of P. triticina have not been discovered yet. The best recognized and characterized of these genes was Lr34 Dyck (1987) that was detected in wheat genotypes around the world (Kolmer et al. 2008).

Polyphenol oxidase (PPO) and peoxidase (POD) isozymes

The biochemical mechanism involved in plant diseases resistance is a complex process. Because long time total phenols and phenolics have been considered as a primary defense mechanism, whose levels are higher in the resistance cultivars of many crops, such as wheat (Saini et al. 1988; Onyeneho and Hettiarachchy 1992). The present study, isozyme activities were determined depending on electrophoresis of PPO and POD on native-PAGE, which revealed differences in the number of bands between the resistance and the healthy wheat plants at seedling stage (Figure 2). PPO isozyme patterns displayed a total of ten bands at different Rf values varying from 0.125 to 0.901, whereas five bands were polymorphic and the other five bands with Rf values (0.158, 0.505, 0.700, 0.809 and 0.870) were found to be monomorphic (Figure 2). For isoperoxidase appeared a total of seven isoforms with different Rf values ranging from 0.125 to 0.901. Four monomorphic bands with Rf (0.125, 0.700, 0.809 and 0.870) were present in all the leaf rust resistance wheat lines under the greenhouse conditions, as well as in the healthy (Figure 2). On the other hand, the highest number of PPO and POD isozyme markers was recorded in leaf rust resistance wheat lines No. 6, 8 and 10 (three isoforms), while the lowest number was found in the resistance wheat lines No.1, 4, 7 and 9 (one) (Table 3). It was observed that, a positive correlation between number of PPO and POD bands and enzyme activity. Our results were agree with those obtained by Flott et al. (1989); Alejandro et al. (2009); Bariya and Thakkar (2012) mentioned that appearance of new bands of POD in resistance wheat varieties after inoculation with fungal pathogens was also reported by many other authors. Other similar studies mentioned that the isozyme technique is a useful method for selection resistance cultivars. Wheeler et al. (1971); Johnson and Cunningham (1972) showed that peroxidase activity was higher in leaf rust resistant wheat than the healthy. Yang et al. (1984) observed higher POD activity in a resistance wheat variety than a susceptible variety with Erysiphe graminis f. sp. tritici. In addition, they scored the highest number of peroxidase isoforms in the resistant wheat cultivar, which could be the expression of a resistant gene.

Inter-simple sequence repeat (ISSR-PCR) profiles

Eight anchored ISSR primers were screened to identify polymorphic bands between the leaf rust resistance and healthy wheat plants at the seedling stage (Figure 3 and Table 4). The number of fragments amplified per primer ranged from seven (primer ISSR-4) and 24 (primer ISSR-3). A total of 104 scorable fragments were amplified of which 54 monomorphic and 46 bands were polymorphic resulting in a polymorphism of 44.23%. The extent of polymorphism per primer ranged from 9.09% (primer ISSR-5) to 83.33% (primer ISSR-3). On the other hand, primer ISSR-3 scored the highest number of unique markers (four), followed by primers ISSR-4 and ISSR-8 recorded three specific bands. In addition, Primers ISSR-1 and ISSR-2 appeared one specific band, whereas missing in the healthy control. However, primers ISSR-5, ISSR-6 and ISSR-7 have not generated any markers (Tables 4 and 5). The maximum number of specific markers scored in leaf rust resistance wheat line No. 10 (three markers), followed by leaf rust resistance wheat line No. 2, 3, 4 and 8 (two). The minimum number of specific bands existed in the resistance line No. 6 (one) (Table 5). In future these polymorphic markers could be used in discriminating between the leaf rust resistance and susceptible wheat lines in marker assisted selection programs. By knowing the reliable primers to apply and thus obtaining ultimate results, we can test lines of wheat with confidence and improve the selection process. Besides, a backcross program was applied by using

as recurrent parents between high-quality susceptible wheat and resistant gene carrying ones as donor parents. The linage of various cross combinations was screened by using ISSR markers closely linked to Lr genes. These results were in an agreement with Esmail et al. (2015) detected of leaf rust resistance wheat lines by using the Sequence tagged site (STS) marker for resistance genes Lr1 (560 bp) and Lr24 (700 bp), Lr9 (1,100 bp) and Lr47 gene (282 bp). Purnhauser et al. (2000) Purnhauser et al. (2000) mentioned that microsatellite markers (Simple sequence repeats–SSR) were utilized for resistance genes Lr3bg and Lr18. Gupta et al. (1999) showed that application of molecular markers for example, ISSR will be increasing the effectiveness of conventional plant breeding by using markers linked to the trait of interest. Consequently, there was a need to apply a fast method to select the leaf rust resistance cultivars. Molecular markers that were closely linked to target alleles current a useful method in plant breeding because they can help to detect of the resistant genes of interest without the need of performing field evaluations. As well, it allows for screening a large number of breeding plants at early growth stages through short period. In this study, showed that ISSR marker could be used to characterize molecular markers linked to resistant genes of leaf rust (Lr) as an indicator for resistant in wheat breeding programs. In spite of, a large number of molecular markers are available at present, few has yet been done about their practical use in wheat breeding programs. Furthermore, being the genome of common wheat very complicated some molecular markers such as STS, Sequence characterized amplification region (SCAR) maybe give false-positive answers about the existence of the targeted gene, particularly considering the various genetic backgrounds of the lines used either as donor or recipient parents (Blaszczyk et al. 2004). The expression of resistance genes was identified to be modified through the genetic background of a cultivar Gupta et al. (1984) in particular when these genes were transferred in common wheat of related species (Friebe et al. 1996). The introgression of resistance genes could be confirmed by phytopathological experiments as well to prove their phenotypic expression in a new genetic background, discarding modifications for the existence of suppressors. Nocente et al. (2007) observed that conventional cereal breeding is time depleting and based on environmental conditions. The application of molecular markers in breeding programs will allow improving efficiency of selection at an early stage, also by detecting of a single resistance gene in a complex background of other resistance genes. New selected lines will be available, a useful for further breeding programs. Consequently, use of resistant cultivars is the best way to leaf rust control.

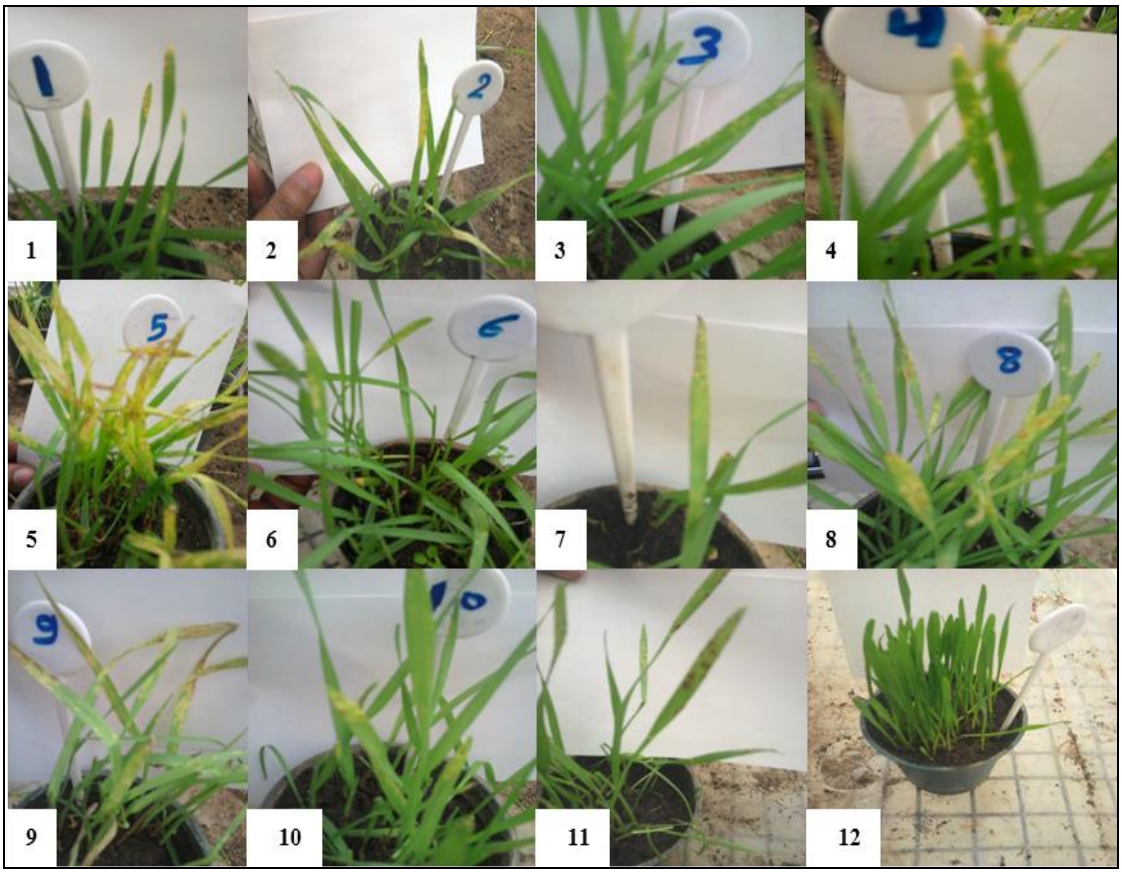
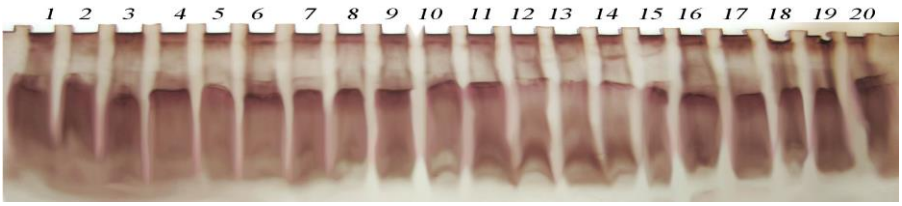
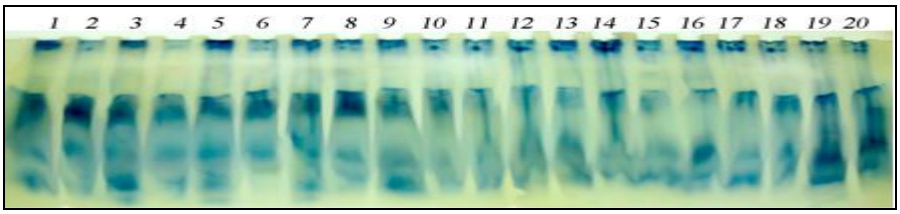


Figure 1. Manifestation of leaf rust pathotype in ten wheat lines at the seedling stage compared with positive (No. 11) and the healthy control (No. 12) under the greenhouse conditions.



PPO



POD

Figure 2. PPO and POD isozyme patterns of leaf rust resistance ten wheat lines compared with the healthy control at the seedling stage.
Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 the healthy plants of wheat lines No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.
Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 the leaf rust resistance wheat lines No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.

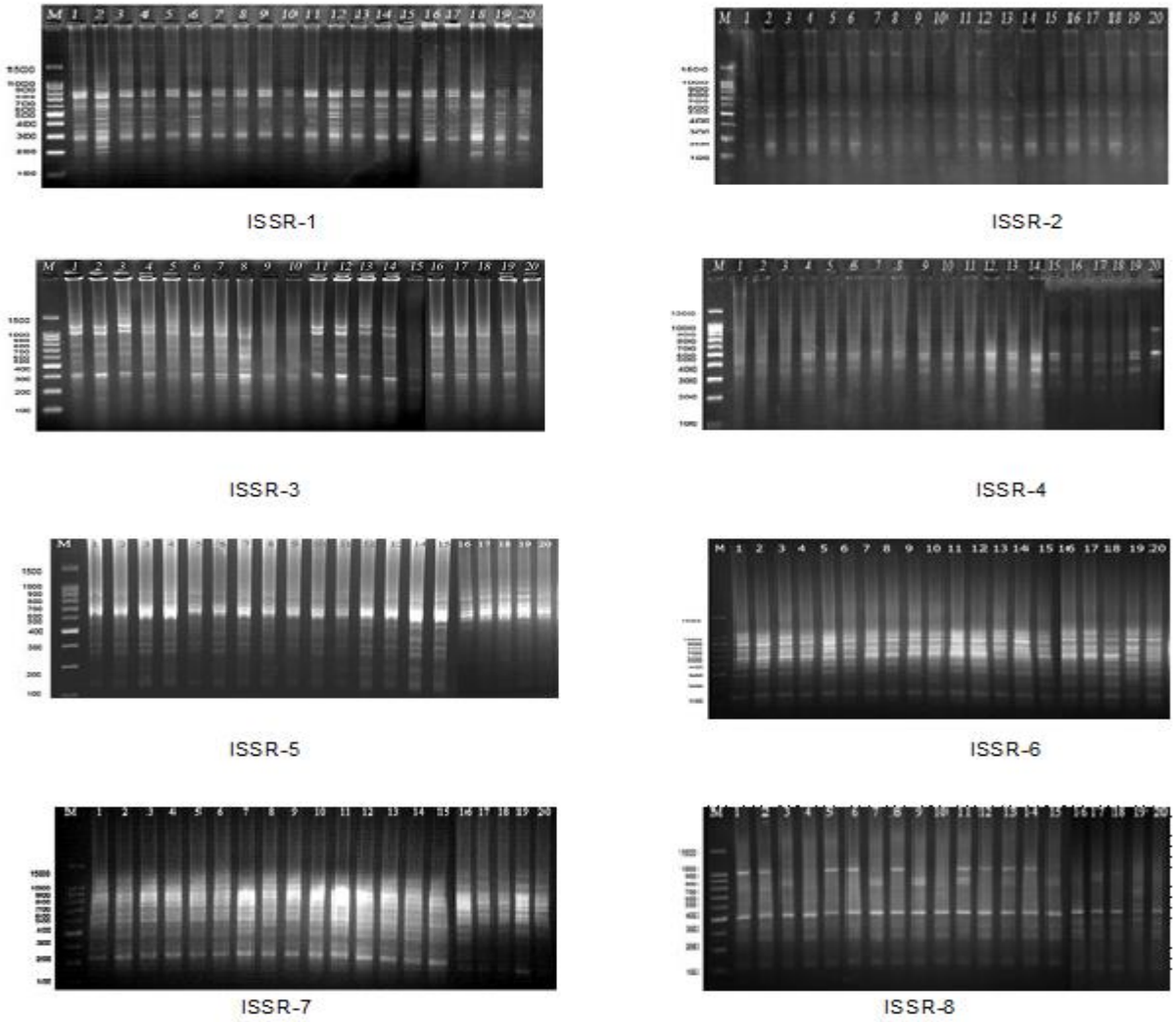


Figure 3. ISSR-PCR profiles using eight anchored primers of leaf rust resistance ten wheat lines compared with the healthy control.
Lane M = 100 bp DNA ladder.
Lanes 1, 3, 5, 7, 9, 11,13, 15, 17, 19 the healthy plants of wheat lines No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.
Lanes 2, 4, 6, 8, 10, 12,14, 16, 18, 20 the leaf rust resistance wheat lines No. 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10, respectively.

Table 1. Major infection type for ten wheat lines inoculated with leaf rust races at seedling stage under the greenhouse conditions.

No. code	Infection type	Host Response	Symptoms
1	1	Resistant	Small uredia with necrosis
2	1	Resistant	Small uredia with necrosis
3	0;	Very resistant	Hypersensitive flecks
4	0;	Very resistant	Hypersensitive flecks
5	1	Resistant	Small uredia with necrosis
6	1	Resistant	Small uredia with necrosis
7	1	Resistant	Small uredia with necrosis
8	2	Moderately resistant	Small to medium sized uredia with green islands and surrounded by necrosis or chlorosis
9	2	Moderately resistant	Small to medium sized uredia with green islands and surrounded by necrosis or chlorosis
10 (Gemeza 11)	2	Moderately resistant	Small to medium sized uredia with green islands and surrounded by necrosis or chlorosis

Table 2. Response of ten wheat lines to leaf rust disease during the adult plant stage.

No. code	The percentage of leaf rust infection	Leaf rust reaction
1	66%	Susceptible
2	66%	Susceptible
3	11%	highly resistance
4	66%	Susceptible
5	66%	Susceptible
6	66%	Susceptible
7	22%	highly resistance
8	22%	highly resistance
9	66%	Susceptible
10 (Gemeza 11)	66%	Susceptible

Table 3. PPO and POD-isozyme markers of leaf rust resistance ten wheat lines at seedling stage.

PPO Rf	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
0.158	+									
0.263										+
0.275						+		+	+	
0.901								+		
Total of bands = 4	1	0	0	0	0	1	0	2	1	1
POD										
0.158						+	+			+
0.275				+		+		+		+
Total of bands = 2	0	0	0	1	0	2	1	1	0	2
Total of PPO and POD bands = 6	1	0	0	1	0	3	1	3	1	3

Rf =Relative mobility L= Line PPO= polyphenol oxidase POD=peroxidase + =Presence of band

Table 4. Polymorphism of the ISSR-PCR primers of leaf rust resistance ten wheat lines at seedling stage.

Primer Code No.	Sequence (5`→3`)	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	Unique bands	% Polymorphism	Molecular sizes of unique markers (bp)
ISSR-1	$(CA)_6AC$	205-3010	19	7	8	1	42.11	1450
ISSR-2	$(CT)_7AC$	210-2200	10	4	6	1	60	1100
ISSR-3	$(CT)_8GC$	200-2500	24	4	20	4	83.33	2500; 1150; 1056;225
ISSR-4	$(CAC)_3GC$	290-900	7	3	4	3	57.14	900; 605; 300
ISSR-5	$(GA)_8YC$	245-860	11	10	1	0	9.09	0
ISSR-6	$(AG)_8T$	340-1100	12	10	2	0	16.67	0
ISSR-7	$(AG)_8C$	325-1300	10	10	0	0	0	0
ISSR-8	$(AGC)_6C$	225-1050	11	6	5	3	45.45	1050; 900; 860
Total		200-3010	104	54	46	12	44.23	11.54%

Table 5. ISSR-PCR markers of leaf rust resistance ten wheat lines at seedling stage.

Primer No.	Unique markers (bp)	Lines (L)									
		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
ISSR-1	1450						+				
ISSR-2	1100										+
	2500										+
	1150								+		
ISSR-3	1056								+		
	225		+								
	900										+
ISSR-4	605				+						
	300		+								
	1050				+						
ISSR-8	900			+							
	860			+							
Total = 12		0	2	2	2	0	1	0	2	0	3

(+)= presence of band.

Conclusions

Leaf rust resistance wheat lines can be used in wheat breeding programs by a backcross program was carried out by using as recurrent parents between the susceptible high-quality wheat line and the carrying the resistant gene ones as donor parents. The progenies of different cross combinations were selected by both resistant tests and marker assisted selection using ISSR closely linked to Lr genes.

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