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DETERMINATION OF RUST RESISTANCE GENES IN PAKISTANI BREAD WHEATS

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Abstract

Stripe and leaf rusts are the major constraints to bread wheat production in Pakistan. Molecular markers were used to investigate the presence of leaf rust and stripe rust resistance gene cluster *Lr34/Yr18* and stem rust resistance gene *Sr2* in 52 Pakistani bread wheat cultivars/lines. PCR amplification of DNA fragments using DNA marker *csLV-34* showed that 13 of the studied cultivars/lines, namely '03FJ26', 'NR 337', 'NR 339' 'NR 347', 'NR 350', 'Manthar', 'Margalla 99', 'Iqbal 2000', 'Saleem 2000', 'Wafaq 2001', 'Marwat 2001', 'Pirsabak 2004' and 'Fareed 2006' carry leaf rust and stripe rust resistance genes *Lr34/Yr18*. Stem rust resistance gene *Sr2* was observed in 36 Pakistani spring wheat cultivars/lines using stm560.3tgag marker. The slow rusting gene *Sr2* needs to be combined with additional stem rust resistance genes to establish durable resistance against *Ug99* in modern wheat cultivars. Low frequency of *Lr34/Yr18* was found in Pakistani wheats. This gene cluster needs to be incorporated into Pakistani wheats for durable rust resistance.

Introduction

Wheat (*Triticum aestivum* L.) production is subjected to many yield limiting biotic and abiotic stresses globally. Among biotic stresses, three rust diseases of wheat have been the most devastating throughout the world including Asia (Singh *et al.,* 2004). Stem (or black) rust (caused by *Puccinia graminis*), was effectively controlled with adoption of the stem rust resistant semi-dwarf spring wheats of the green revolution in South and West Asia during 1960s (Duveiller *et al.,* 2007). However, the recent threat of the evolution of *Ug99* pathotype of stem rust in East Africa and its migration to Arabian Peninsula and Nile Valley is becoming a serious threat to wheat production in Asia (Singh *et al*., 2004). Stripe rust caused by *Puccinia striiformis* and leaf rust caused by *P. triticina* had been and continue to be the major production constraints in Asia and rest of the world. These two fungal diseases of wheat have caused huge production losses in Asia, America, Australia and other parts of the world. According to Singh *et al*., (2004), stripe and leaf rust could adversely affect wheat production in Asia by 46 and 63%, respectively, if susceptible wheat cultivars are grown.

Wheat rusts can be controlled to some extent with the application of fungicides. However, this raises an environmental concern and also increases the cost of production. The latter may not be an affordable option for resource poor farmers with small land holdings. Therefore, the use of resistant cultivars is probably the most economical, efficient and environment and farmer friendly strategy to minimize yield losses by rusts (Ittu, 2000). The utilization of race-specific type of resistance has dominated wheat breeding for the last 50 years. This type of resistance is conferred by a single or few major genes and is associated with hypersensitive response of host plant cells to infection of rust. However, the resistance provided by these genes can be short-lived as new races of the rust pathogens are continuously evolving and hence acquiring virulence to these genes (Stubbs, 1985). An alternative to solve this problem is to deploy genetic diversity for resistance in new cultivars (William *et al.,* 2003) and breed for durable resistance (Singh *et al.,* 2004). Pathotype non-specific resistance conditioned by minor genes results in reduced rate of disease development (Bariana *et al.,* 2007). Such type of resistance may continue for a relatively longer period of time as the pathogen needs not to change its type for survival. Race-non-specific resistance, conferred by minor genes with additive effect, often provides slow rusting (Caldwell, 1968) at adult plant stage. Combing genes of such nature in one wheat cultivar is suggested to achieve near immunity to rust diseases (Singh *et al.,* 2000).

Gene-for-gene specificity between host resistance genes and different avirulence genes in pathogen can be employed for postulation of resistance genes in host plant. However, this method is best suited for seedling resistance genes because the interaction between resistance genes and stage of development of plant at which these genes express can obscure the gene postulation (Kolmer, 1996). These problems can be overcome by using DNA-based markers to identify resistance genes (McCartney *et al.,* 2005). Rust resistance genes in Pakistan have mostly been postulated using multi-pathotype tests. Tariq-Khan *et al*., (2012) screened subsets of synthetic hexaploid wheat to detect the presence of adult plant stripe rust resistance genes in field. Kazi *et al*., (2012) conducted seedling tests to screen 95 synthetic wheats against prevalent races of stripe rust in Pakistan under glasshouse conditions. Rasheed *et al*., (2012) characterized leaf rust resistance genes *Lr10*, *Lr17a* and *Lr27+31* with the help of DNA markers in a F_2 population obtained from crossing a leaf rust resistant and leaf rust susceptible Pakistani wheat variety. Mustafa *et al*., (2013) characterized 38 Pakistani commercial varieties through DNA markers for the presence of six leaf rust resistance genes. However, very few studies have been conducted to determine the presence/absence of major stripe and stem rust resistance genes in Pakistani wheat genotypes. This study was, therefore, aimed to identify adult plant (durable) leaf and stripe rust resistance

gene complex *Lr34*/*Yr18* and adult plant stem rust resistance gene *Sr2* in 52 Pakistan bread wheat cultivars/lines.

Materials and Methods

Plant materials: A total of 52 Pakistani wheat cultivars and advanced breeding lines were used to investigate the presence of leaf rust and stripe rust resistance gene cluster *Lr34*/*Yr18* and stem rust resistance gene *Sr2* by DNA markers. Seeds of 46 wheat cultivars/lines were provided by Coordinated Wheat, Barley & Triticale Program, National Agricultural Research Centre, Islamabad, Pakistan, whereas seeds of 6 advanced lines were provided by Barani Agricultural Research Station, Fateh Jang, Pakistan. The near isogenic lines (NILs) 'Avocet' and 'Avocet+*Yr18*' were used as negative and positive check, respectively for *Lr34*/*Yr18* gene complex. Wheat cultivar 'Hartog' was used as positive check for *Sr2*.

DNA Isolation and PCR analysis: DNA was extracted from the mature dry seeds of all cultivars/lines using a standard protocol. Four seeds of each cultivar/line were ground using a crushing machine and put into a 1.5 ml Eppendorf tube. 800µl of warmed (65^{o}C) CTAB buffer was added to each tube and the resulting mixture was vortexed thoroughly to homogenize. The tubes were incubated in a water bath for 40 minutes at 65ºC and then left for five minutes at room temperature. Chloroform and Isoamyl alcohol (24:1) was added to each tube and the solution was mixed gently by inverting tubes for two minutes. The samples were centrifuged at 13,200 rpm for 15 minutes. Supernatant was taken and collected in new 1.5 ml Eppendorf tubes. Cold Isopropanol (2/3rd volume of supernatant) was added to each tube and mixed gently. Samples were centrifuged at 14,000 rpm for 10 minutes to precipitate the DNA. The supernatant was discarded; the DNA pellets were washed with 70% ethanol and subsequently air dried at room temperature for 10 minutes. The pellets were dissolved in 100µl TE buffer (pH 8.0) and treated with 1µl RNase-A (10mg/ml) for 1-2 hours at 37^{o}C.

Polymerase chain reaction (PCR) using primer pair *csLV-34* (Table 1) was performed for identification of leaf rust and stripe rust resistance gene cluster *Lr34/Yr18,* following Lagudah *et al*., (2006), whereas stem rust resistance gene *Sr2* was identified using the primer pair *stm560.3tgag* (Table 1). PCR products of primer pair *csLV34* were electrophoresed on 2% agarose gel and visualized under UV light after staining with ethidium bromide. PCR products of primer pair *stm560.3tgag* were resolved using 5% Polyacrylamide Gel Electrophoresis (PAGE). Wheat cultivars/lines parallel to 'Hartog'" in the PAGE image was scored as positive, whereas any stuttering up or below the bands of 'Hartog' as negative (Urmil Bansal, personal communications).

Results and Discussion

Amplification of genomic DNA of 52 Pakistani spring wheat cultivars/lines using *csLV34-F* and *csLV34-R* primers (*Lr34/Yr18*) yielded a PCR product of 150bp similar in size to that of the positive control Av-*Yr18* in eight of the cultivars/lines. This indicated that these cultivars/lines (Table 2) carry leaf rust and stripe rust resistance gene complex *Lr34/Yr18*. Thirty six cultivars/lines yielded a PCR product of 229bp similar in size to that of the negative control 'Avocet', indicating that these possess the recessive allele at *Lr34*/Yr18 locus (Table 2). Three cultivars/lines failed to amplify any PCR product using csLV34 primers. Primer pairs of *stm560.3tgag* produced a PCR product of 171bp similar in size to that of positive control 'Hartog' in 36 cultivars/lines (Table 2). This indicated that these cultivars/lines possess stem rust resistance gene *Sr2*. Most of the cultivars that carried *Lr34*/*Yr18* gene cluster also had *Sr2* (Table 2; Figs. 1-2).

The present study demonstrated that very few Pakistani wheat cultivars carry leaf rust resistance gene *Lr34* and stripe rust resistance gene *Yr18*. Our results were similar to Singh *et al*., (1999) who also found a low frequency of *Lr34/Yr18* (only in 7 of the 163) in wheat cultivars of China. Using *csLV34* marker, Liang *et al*., (2009) found *Lr34/Yr18* in 55 of the 263 CIMMYT wheat lines they studied. A relatively higher frequency of *Lr34/Yr18* (28.3%) was observed in Hungarian wheats using *csLV34* marker by Wang *et al*., (2009). The low frequency of *Lr34/Yr18* indicates that this gene cluster has not been incorporated into Pakistani wheat cultivars by wheat breeders. The use of this adult plant resistance gene cluster *Lr34/Yr18* with other minor genes for resistance can confer near immunity to leaf and stripe rust infection (Singh *et al.,* 2000). In a conducive environment where susceptible cultivars exhibited 100% leaf rust severity, the cultivars possessing only *Lr34* showed 40% severity and cultivars carrying *Lr34* with two or three additional genes displayed 1-5% severity (Singh *et al.,* 2004). Leaf rust resistance gene *Lr34* has been found still conferring resistance to leaf rust in wheat cultivars 'Mentana' and 'Ardito' released in the beginning of the last century (Lagudah *et al.,* 2009). This indicates the effectiveness of *Lr34* in conferring resistance to wheat leaf rust. Therefore, this gene cluster may provide durable source of resistance against both leaf and stripe rust resistance in Pakistan.

Table 1. Primer sequences and expected PCR products of DNA markers closely linked with rust resistance genes.

Gene	Marker	Primer sequence	Base pairs	Reference
Lr34/Yr18	csLV34-F	GTTGGTTAAGACTGGTGATGG	150	Lagudah *et al*., (2006)
	csLV34-R	TGCTTGCTATTGCTGAATAGT		
Sr2	stm560.3tgag-F	GGAGGGAAACTATCAAAATATGCTGGT	171	Bansal *et al*., (2010)*
	stm560.3tgag-R	GTTGGTTGATAAATCCAGTTTGGCAA		

*Primer sequences and expected product sizes provided by U.K. Bansal (University of Sydney, Australia)

Table 2. Occurrence of stem rust (*Sr2*) and Leaf and Stripe rust (*Lr34/Yr18*) resistance genes in Pakistani bread wheat cultivars/lines.

No.	Cultivar/Line	Parentage	*Sr2*	*Lr34/Yr18*
1.	99FJ03	PFAU/SERI/BOW	+	M
2.	03FJ26	PASTOR/3/MUNIA//CHEN/ALTER84/5/CNDO/R143	+	+
3.	04FJS35	N/A	-	-
4.	05FJS3023	N/A	-	-
5.	06FJ05	N/A	+	-
6.	06FJ10	N/A	+	-
7.	NR-337	DORADE-5/WAFAQ 01	+	+
8.	NR-338	PARUS/PASTOR	-	-
9.	NR-339	OTUS/TOBA97	+	+
10.	NR-340	PIFED/DHARWAR DRY	+	-
11.	NR-341	ACC.8528/INQALAB 91	-	-
12.	NR-342	PBW65/2*PASTOR	-	-
13.	NR-343	PBW65/2*PASTOR	+	-
14.	NR-344	WBLL1*2/VIVITSI	+	-
15.	NR-345	WBLL4/KUKUNA//WBLL1	+	-
16.	NR-346	KAUZ//ALTAR 84/AOS/3/MILAN/KAUZ/4/HUITES	-	-
17.	NR-347	OASIS/SKAUZ//4*BCN*2/3/PASTOR	+	+
18.	NR-348	SITE/MO/3/VORONA/BAU//BAU	-	-
19.	NR-349	CROC_1/AE.SQUARROSA (213)//PGO/3/UP2338	-	-
20.	NR-350	SST 57/INQALAB 91	-	+
21.	NR-351	PASTOR//HXL7573/2*BAU	+	-
22.	NR-352	ATTILA/PASTOR	+	-
23.	NR-353	SERI*3//RL6010/4*YR/3/PASTOR/4/BAV92	+	-
24.	TD-1	PASTOR/OPATA/3/BOW/PRL/BUC	+	-
25.	Avocet	Thatcher-Agropyron elongatum translocation/3 *Pinnacle//WW15/3/Egret	-	-
26.	Avocet Y*r18*	N/A	+	+
27.	V-03079	PBW65*2/PASTOR	+	-
28.	V-03138	LUAN/KOH-97	+	-
29.	V-04188	PRL/2*PASTOR	+	-
30.	V-04189	PBW65/2*PASTOR	+	-
31.	Manthar	KAUZ//ALTAR84/AOS	+	+
32.	Tatara	ATTILA	-	-
33.	Bhittai	VEE/TRAP#1//SOGHAT-90	-	-
34.	Fakhre Sarhad	KVZ/BUHO//KAL/BB	+	-
35.	Zarlashta 99	URES/BOW`S'	+	-
36.	Inqalab 91	WL 711/CROW`S'	+	-
37.	Margalla 99	OPATA/BOW'S'	+	+
38.	Iqbal 2000	BURGUS/SORT 12-13//KAL/BB/3/PAK 81	+	+
39.	Saleem 2000	CHAM6//KITE/PGO	+	+
40.	Marvi 2000	CMH-77A917/PKV 1600//RL6010/6*SKA	-	-
41.	Auqab 2000	CROW'S'/NAC//BOW'S'	+	-
42.	Haider 2000	CHIL/WUH3	-	-
43.	Chenab 2000	CATBIRD	+	M
44.	Wafaq 2001	OPATA/RAYON//KAUZ	+	+
45.	Marwat 2001	WL 711/HD 2169//GHSK`S'	+	+
46.	Moomal 2002	BUC'S'/4/TZPP/IRN46	+	-
47.	GA 2002	DWL 5023/S N B//SNB	-	-
48.	AS 2003	KHIP/31708//CM74A370/3/CIAN O79/4/RL6043/4*NAC	-	M
49.	SH 2003	INQALAB-91/FINK'S'	+	-
50.	Pirsabak 2004	KAUZ/PASTOR	-	+
51.	Pirsabak 2005	MUNIA/CHTO//AMSEL	+	-
52.	Fareed 2006	PTS/3/TOB/LFN/BB/HD-832-5//ON/5/G-V/ACD 'S'//HPO 'S'	+	+
53.	Shafaq 2006	V87094(LU26/HD2179)	+	-
54.	Seher 2006	CHILL/2*STAR/4/BOW//BUC/PVN	+	-

Note: + = Resistant gene is present; - = Resistant gene is absent; M = Missing, no amplification

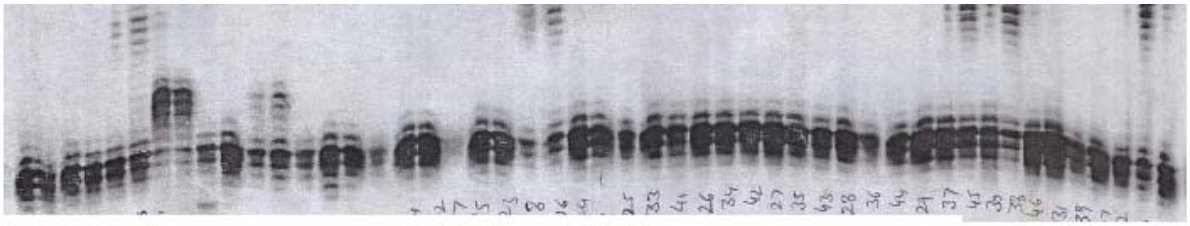

Fig. 1. Electropherogram of PCR products amplified with *stm560.3* marker for *Sr2* gene in Pakistani wheat cultivars/lines (Numbers and signs are as given in Table 2). The + indicates the presence of *Sr2* resistant gene, while - indicates the absence of *Sr2* resistant gene in wheat cultivars/lines.

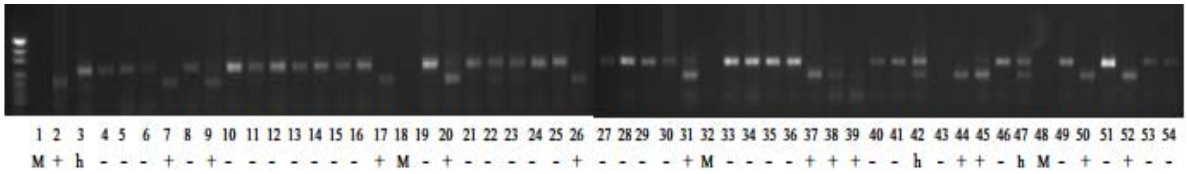

Fig. 2. Electropherogram of PCR products amplified with *csLV34* marker for *Lr34/Yr18* gene in Pakistani wheat cultivars/lines (Numbers and signs are as given in Table 2). The + indicates the presence of *Lr34/Yr18* resistant gene, - shows the absence of *Lr34/Yr18* resistant gene, while M indicates no amplification of PCR product in wheat cultivars/lines.

One of the most widely adapted wheat variety of Pakistan, 'Inqalab-91', does not carry *Yr18*. This variety has become susceptible to stripe rust due to the breakdown of resistance of *Yr27* gene (Duveiller *et al.,* 2007). Wheat cultivar 'Inqilab-91' occupied about 6 million hectares of area under wheat in Pakistan upto 2007-08. This variety has been banned for cultivation in some parts of Pakistan. Incorporation of *Yr18* gene into wheat cultivars may help to reduce the incidence of stripe rust in Pakistan. Marker assisted selection will greatly facilitate the transfer of these into adapted wheat cultivars in this region. Leaf tip necrosis (*Ltn*) is also strongly correlated with the presence of *Lr34* gene. However, selection based on the *Ltn* can sometimes be misleading, as *Ltn* is a multi-genic trait whose expression varies with environment (Lagudah *et al.,* 2009). Wheat lines with *Ltn* phenotype but lacking *Lr34* gene has been identified (Lagudah *et al.,* 2009).

Stem rust resistance gene *Sr2* was found in the 36 of the 52 Pakistani wheat cultivars/lines studied. The high frequency of *Sr2* in Pakistani wheat is probably due to the introduction of semidwarf wheat cultivars resistant to stem rust from CIMMYT during and after green revolution. Singh *et al*., (2008) also found a high frequency (92%) of *Sr2* gene in wheat genotypes included in the 22nd SAWSN CIMMYT nursery using *stm560.3tgag* marker. This gene confers race-non-specific response and is associated with variable level of disease symptoms influenced by genetic background and environmental conditions. *Sr2* is one of the most important genes of stem rust that grants adult plant resistance and is used in modern plant breeding (McIntosh *et al.,* 1995). Its recessive inheritance nature makes selection in a breeding programme more complicated (Mishra *et al.,* 2005). The presence of this gene is associated with phenotypic marker pseudo-black chaff, a distinctive spike and stem blacking melanism pigmentation in adult plant (Hare & McIntosh, 1979) and leaf chlorosis in seedling (Brown, 1997). The pseudo-black chaff can assist selection for *Sr2* but it is not a reliable marker, as its expression depends on genetic background and environmental conditions, and its levels are believed to be negatively correlated with grain yield (Hare & McIntosh, 1979).

The resurgence of stem rust pathotype *Ug99* is alarming because it signals the breakdown of stem rust resistance that protects wheat cultivars in many countries. Although there are several genes that confer resistance to this pathotype, the long term strategy may focus on combination of adult plant resistance gene *Sr2* with other additive genes of similar nature to activate long term durability. Further more, the linkage of this marker with *Sr2* can be broken (Mishra *et al.,* 2005). Use of this phenotypic marker in breeding for stem rust needs more caution, so marker assisted breeding may be useful to facilitate selection for this durable resistance gene.

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