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A PERSPECTIVE OF LEAF RUST RACE FHPRN AND ITS IMPACT ON LEAF RUST RESISTANCE IN PAKISTANI WHEAT VARIETIES

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Abstract

Leaf rust infected leaves of a widely growing variety Seher-06 were collected in wheat season of 2011-12. The leaf rust isolates were assessed on Thatcher derived *Lr* isogenic lines and a race FHPRN was identified. Seventy six wheat varieties/lines besides *Lr* isogenic lines were screened against this race for seedling in glass house and for adult plant resistance at Bahawalpur and Faisalabad during 2012-13. *Lr*1, *Lr*2a, *Lr*9, *Lr*19, *Lr*24, *Lr*10+27+31 (Gatcher) and *Lr*28 were found completely resistant at both stages against FHPRN. Molecular screening of the wheat varieties/lines indicated the presence of leaf rust resistance genes *Lr*9 (0%), *Lr13* (43%), *Lr*19 (1%), *Lr*20 (0%), *Lr*24 (4%), *Lr26* (23%), *Lr*28 (0%), *Lr34* (38%), *Lr*37 (1%) and *Lr*47 (1%) in them. Field data suggested that As-02 (*Lr*10+26+34), Bhakar-02 (*Lr*13) and Shafaq-06 (*Lr*10+13+27) were resistant; Pasban-90 (*Lr*10+13+26+27), Chenab-2000 (*Lr*10+13+26+27+31+34), Fbd-08 (*Lr*10), Millat-11 (unknown) and Punjab-11 (unknown) were found moderately resistant; Blue silver (*Lr*13+14a), Pak-81 (*Lr*10+23+26+31), Bahawalpur-97 (*Lr*13+26) and Lasani-08 (*Lr*13+27+31) were susceptible while Sh-88 (unknown), Auqab-2000 (*Lr*10+23+26+27+31), Iqbal-2000 (*Lr*3+10+13+26+27+31), Bahawalpur-2000 (*Lr*34) and Seher-06 (*Lr*10+27+31) were found highly susceptible against FHPRN. Present and previous studies revealed the presence of *Lr*3, 10, 13, 14a, 23, 26, 27, 31 and 34 in the Pakistani wheat varieties yet lacking *Lr*9, 19, 24 and 28. Therefore, the latter genes and their effective combinations should be incorporated in Pakistani varieties to combat leaf rust effectively.

Key words: Leaf rust race, FHPRN, Resistance, Wheat

Introduction

Wheat being a staple food is cultivated in many parts of the country. Among 50 known diseases on wheat, rusts, smuts and powdery mildew are most destructive and frequently found diseases in Pakistan (Rattu *et al*., 2011; Qamar et al. 2014). Among wheat rusts, leaf rust covers 80% area of the cultivated land in Pakistan followed by stripe rust on 70% area (Singh *et al*., 2005; Afzal *et al*., 2008; Ibrahim et al. 2013) while stem rust occurs only in parts of Sindh, South Punjab and Kaghan with low intensity. Wheat leaf rust caused by *Puccinia triticina* Eriks., is a common disease and it can cause up to 50% yield losses (McIntosh *et al*., 1995). In Asia, leaf rust posses a major threat to wheat where India and Pakistan are main wheat producing countries (Singh *et al*., 2004). Several records of rust epidemics in wheat producing areas of South Asia reveal its importance in this region (Hassan *et al*., 1973; Hassan, 1979; Hussain *et al*., 1980). In response the Pakistani breeders have introduced many high yielding and disease resistant wheat varieties during Pre-green revolution era–up to 1966, Green revolution era (1967-1977) and Post green revolution era (1977 onward). However, the rust pathogen has continued to evolve and defeated many wheat varieties in Pakistan.

Puccinia triticina reproduces asexually and has the ability to evolve new virulent races through mutation which overcome specific *Lr* genes. To mitigate this would require a continuous monitoring of the host and the pathogen. Abbas *et al*. (2009) identified a series of leaf rust pathotypes in Pakistan viz., 104-1, 2, 3, 6, 7, 76-1, 3, 5, 10, 12, 10-1, 3, 9, 10, 11, 12, 104-2, 3, 6, 7 but in last five years no such report on leaf rust pathotypes has appeared from Pakistan. Presently breeders are using the North American nomenclature by Long and Kolmer (1989). Rapid evolution of pathotypes (Wellings *et al*., 2000) needs an intensive research work to improve genetic diversity in host that can reduce the risk of pandemics (Joshi *et al*., 2011). Using disease resistant wheat varieties is an ecologically advantageous method (Vanzetti *et al*., 2011). However if one gene is widely used in breeding, the resulting cultivars may quickly lose resistance because of the appearance of new pathogen races (Tyryshkin *et al*., 2006). Among 70 *Lr* genes (McIntosh *et al*., 1995; 2007; 2012; Rasheed *et al*., 2011), most confer seedling resistance (McIntosh *et al.,* 2008; Sun *et al.,* 2009; Samsampour *et al.,* 2010) and few genes confer adult plant resistance (APRs). Seedling resistance genes have a life span of 5-6 years (Singh *et al*., 2005) against pathogen diversity while APRs can provide more durable resistance but in combination they both can be more effective. During last two decades, many of the *Lr* genes have been defeated against *P. triticina* isolates of Pakistan viz., *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr9*, *Lr10*, *Lr13*, *Lr15*, *Lr17*, *Lr20*, *Lr23*, *Lr24*, *Lr26* and *Lr29* (Mirza *et al*., 2000) while *Lr19* and *Lr28* remained effective (Mirza *et al*., 2000; Fayyaz *et al*., 2008; Rattu *et al*., 2009).

Pyramiding of APRs with APRs or with seedling genes can be an effective strategy to enhance durability of wheat resistance to rust (Leonard & Szabo, 2005). Many of the gene combinations have been reported e.g. *Lr*12 and *Lr*13 with *Lr*34 (Roelfs, 1988) and *Lr*10 with *Lr*26 (Fayaz *et al*., 2008), *Lr*24 with *Lr*26 and *Lr*28 (Sohail *et al*., 2014). Gene pyramiding for rust resistance has become an easy approach because of the linked molecular markers to the rust resistance genes. Few of the Pakistani wheat varieties have been screened through molecular markers (Babar *et al*., 2010; Hussain *et al*., 2011; Ejaz *et al*., 2012; Mustafa *et al*., 2013; Sohail *et al*., 2014), still many of them are uncharacterized. Globally, several molecular markers linked with *Lr* genes have been reported in various studies and multiple markers for *Lr*1, *Lr*3, *Lr*9, *Lr*10, *Lr*13, *Lr*19,

*Lr*21, *Lr*23, *Lr*24, *Lr*25, *Lr*27, *Lr*28, *Lr*29, *Lr*31, *Lr*34, *Lr*35, *Lr*37, *Lr*39, *Lr*46, *Lr*47, *Lr*50 and *Lr*51 are now available at http://maswheat.ucdavis.edu. During present study a race of Seher-06 assessed and identified through North American Nomenclature system and was used for seedling and adult plant screening of Pakistani wheat varieties/lines. Thus the present status of *Lr* genes was assessed against this race from Seher-06 in glass house and field conditions. Molecular markers were used to identify effective seedling and APR genes and their combinations thus assessing the impact of the newly developing leaf rust pathotypes/races on existing wheat genetic resources for adopting an effective breeding strategy.

Materials and Methods

Virulence and race analysis: Infected leaves of Seher-06 were collected from nine different sites in three provinces of Pakistan (Table 1) during wheat season of 2011-2012 for virulence and race analysis. Nine pots were sown by seeds of a susceptible variety Morocco for the development of leaf rust inoculum from Seher-06. Seven days old Morocco plants were gently rubbed with infected leaves of each isolate of Seher-06 and placed overnight in a dew chamber at +18°C. Plants were then transferred to glass house at +25°C. At fourteenth day of inoculation single pustule of each isolate was taken from Morocco plants through sterilized spatula and re-inoculated on healthy Morocco plants for further multiplication repeating above mentioned protocol. Seeds of thirty seven *Lr* isogenic lines were sown in nine plastic trays. At two leaf stage all plants in the trays were inoculated with nine single pustule isolates of Seher-06 by making a suspension of urediospores in a light mineral oil carrier (Singh & Rajaram, 1991). The oil was allowed to evaporate from the leaves for 30–60 min and the seedlings were placed overnight in a dew chamber at ± 18°C. They were then transferred to a glass house at ± 25°C. At fourteenth day of inoculation, the lines were scored for infection type (IT) according to the scale of Stakman *et al*. (1962), where 0 corresponds to nearly immune; 1 to very resistant; 2 to moderately resistant; 3 to susceptible and 4 to highly susceptible. Thus a leaf rust race of Seher-06 was isolated and named through North American nomenclature (Long &Kolmer, 1989).

Wheat for seedling/adult plant/molecular screening: Seventeen Pakistani wheat varieties and Fifty nine advance wheat lines were used in this study for seedling, adult plant and molecular screening. All varieties and lines were collected from Crop Diseases Research Programme (CDRP) at NARC, Islamabad; Wheat Research Institute (WRI) at Ayub Agricultural Research Institute, Faisalabad and Genetics lab at Quaid-i-Azam University, Islamabad (Table 3). Wheat germplasm included: National Wheat Disease Screening Nursery (NWDSN), International Maize and Wheat Improvement Center's (CIMMYT) material, prominent Pakistani varieties, synthetic hexaploid wheat lines and some other advance wheat lines. Thirty seven *Lr* isogenic lines were also included in rust screening. Moreover, positive controls of all *Lr* genes and Morocco as negative control were used in molecular screening for maker validation.

Table 1. Collection sites of infected leaves of Seher-06 along with their coordinates.

Sr. No.	Location	Province	Latitude	Longitude	Sr. No.	Location	Province	Latitude	Longitude
1.	Rahim Yar Khan	Punjab	26.1220	68.2259	6	Akora	KPK	34.0006	72.1216
2.	PSC Khanewal	Punjab	30.3231	72.0285	7	Khanewal	Punjab	30.3000	71.9333
3.	PSC Khanewal	Punjab	30.3231	72.0285	8	Shahgarh	Punjab	29.9833	71.0833
4.	Chichawatni	Punjab	30.5333	72.7000	9	Malti	Sindh	25.0430	68.6561
5.	Peshawar	KPK	34.0149	71.5804					

Table 2. PCR amplification conditions optimized for molecular markers of *Lr* genes and their observed product size.

Gene= Marker (type)	PCR programme	Product	Reference
*Lr*9= $SCS5_{550}$	95°C 2min, 30 cycles (94°C 1min, 64°C 1min, 72°C 1min), 72°C 7min, 4°C 10 min	550bp	Gupta *et al*., 2005
*Lr*13 =Xgwm630 (SSR)	94°C 3min, 35 cycles (94°C 1min, 60°C 1min, 72°C 2min), 72°C 10min, 4°C 10min	120/130bp	Seyfarth *et al*., 2000
*Lr*19= EF2/ER4 (EST)	Touchdown 60°C	191bp	(http://wheat.pw.usda.gov)
*Lr*20= 638F/638R (STS)	Touchdown 60°C	542bp	Neu *et al*., 2002
*Lr*24= Sr24#12 (AFLP)	Touchdown 60°C	500bp	(http://wheat.pw.usda.gov)
*Lr*26= Iag95 (SCAR)	95°C 10 min., 35 cycles (94°C 30 sec., 55°C 1 min., 72°C 1.10 min.), 72°C 5 min., 10°C 10 min.	1100bp	Mago *et al*., 2002
*Lr*28= $SCS421_{570}$ (SCAR)	95°C 10 min., 35 cycles (94°C 30 sec., 60°C 1 min., 72°C 1.10 min.), 72°C 5 min., 10°C 10 min.	570bp	Cherukuri *et al*., 2005
*Lr*28= mag3092 (STS)	95°C 10 min., 35 cycles (94°C 30 sec., 52°C 1 min., 72°C 1.10 min.), 72°C 5 min., 10°C 10 min.	200/225bp	Sohail *et al*., 2014
*Lr*34= csLV34 (STS)	95°C 10 min., 35 cycles (94°C 30 sec., 60°C 30 sec., 72°C 30 sec.), 72°C 5 min., 10°C 10 min.	150/229bp	Lagudah *et al*., 2006
*Lr*37= VENTRIUP/LN2 (CAPS)	95°C 10 min., 35 cycles (94°C 45 sec., 65°C 30 sec., 72°C 1.0 min.), 72°C 5 min., 10°C 10 min.	259bp	Helguera *et al*., 2003
*Lr*47= PS10R/PS10L2 (CAPS)	Touchdown 60°C	282bp	Helguera *et al*., 2000

i. Seedling screening: Inoculum of a leaf rust race from Seher-06 was used for screening of seventeen varieties and fifty nine advance wheat lines at seedling stage. Leaf rust infection was developed by following above mentioned protocol. At fourteenth day of inoculation, the varieties/lines were scored for infection type (IT) according to 0-4 scale of Stakman *et al*. (1962). Infection types '0 to 23' were considered as resistant; '3 to 4' as susceptible and 'X' was considered as mesothetic response.

ii. Adult plant screening: Seventeen wheat varieties, fifty nine advance wheat lines and thirty seven isogenic lines of *Lr* genes were grown at two locations viz., Ayub Agriculture Research Institute (AARI), Faisalabad and at Regional Agricultural Research Institute (RARI), Bahawalpur during wheat season of 2012-13. A susceptible wheat variety Morocco was also grown all around the experimental fields for increasing rust severity. At booting stage wheat material was heavily inoculated three times with interval of seven days through inoculum of a race from Seher-06 by mixing it in water. Final rust severity was recorded according to the modified Cobb's Scale (Peterson *et al*., 1948). Infection types 0, R and TR were considered as resistance, TRMR, RMR, MR, TMRMS, MRMS and MSS were considered as moderate type of infection (moderately resistant/susceptible), TS was considered as trace susceptibility while S was considered as highly susceptible response.

iii. Molecular screening

a. DNA extraction and PCR amplification: Genomic DNA was extracted from leaf tissues of seventy six wheat varieties/lines using the modified CTAB protocol described in Bansal *et al*. (2014). DNA was quantified with a Nano Drop 3300 Fluoro spectrometer and diluted to a final concentration of 30ng/µl. Eleven validated and linked molecular markers (Bansal pers. comm.; Sohail *et al*., 2014; Imbaby *et al*., 2014) were used to check the presence of *Lr9*, *Lr13*, *Lr19*, *Lr20*, *Lr24*, *Lr26*, *Lr28*, *Lr34*, *Lr37* and *Lr47* in selected wheat germplasm. A total of 10µl PCR mixture contained 2µl DNA template (30ng/µl), 1µl 10x buffer (containing 15 mM MgCl2), 1µl of each dNTP (2.5 mmol/µl), 0.25µl forward primer (5µM), 0.25µl (5µM) reverse primer, 0.04µl Taq DNA polymerase (0.2U) and 5.5µl ddH_2O. Amplification was performed in T100™ Thermal Cycler (BIO-RAD, USA). PCR conditions were modified and optimized for each marker (Table 2). The amplified products were resolved on 2% agarose gel. The bands were visualized under UV in gel documentation system (Bio Rad).

Results

The race of Seher-06: The leaf rust race FHPRN was observed in nine leaf rust isolates of Seher-06 during wheat season of 2011-2012. The avirulent/virulent formula of FHPRN is: *Lr*1, 2a, 9, 11, 15, 19, 20, 21, 24, 10+27+31, 28, 34, 37/*Lr*2b, 2c, 3, 3ka, 3bg, 10, 12, 13, 14a, 14b, 16, 17, 18, 22a, 22b, 23, 25, 26, 29, 30, 32, 33, 35, 36.

Seedling screening of wheat against FHPRN: At seedling stage, most of the varieties/lines showed susceptible response against FHPRN. Eleven varieties showed susceptibility at seedling stage followed by Lasani-08 with 'X' type response. Five varieties viz., Pak-81, Chenab-2000, Bahawalpur-2000, Bhakar-02 and Shafaq-06 showed resistant response at seedling stage. Similarly, most of the wheat lines also showed susceptible response at seedling stage (Table 3). Among fifty nine wheat lines, twenty eight showed susceptible response followed by thirteen with resistance response, five showed 'X' infection type while the remaining showed no response.

Adult plant screening of wheat against FHPRN: Among thirty seven *Lr* isogenic lines, most showed susceptible response against FHPRN at both locations in field where other races may also exist. Among thirty seven *Lr* genes, only *Lr*1, 2a, 9, 19, 24, 10+27+31 and *Lr* 28 were found resistant followed by moderate response of *Lr*3bg, 12, 17, 22a, 36 and 37 against FHPRN in field. Among seventeen wheat varieties, Chenab-2000, As-02, Bhakar-02 and Shafaq-06 were found resistant, Pasban-90, Iqbal-2000, Fbd-08, Millat-11 and Punjab-11 were moderate (Moderately resistant/susceptible) while three of them showed trace susceptibility (TS) and eight were highly susceptible. Contradictory to varieties, among fifty nine advance wheat lines, seventeen were observed as resistant followed by eleven as moderate, three showed trace susceptibility while the remaining were susceptible.

Molecular screening of wheat varieties/lines: The results and validation of molecular markers of *Lr9*, *Lr13*, *Lr19*, *Lr20*, *Lr24*, *Lr26*, *Lr28*, *Lr34*, *Lr37* and *Lr47* in seventeen varieties and eighty three advance wheat lines have been summarized in Table 3; Fig. 1. All of the markers did not amplify their specific bands in negative control Morocco. Three of the *Lr* genes were frequently found in wheat varieties and lines viz., *Lr13*, 26 and 34. The SCAR marker SCS_{550} linked with *Lr9* only amplified the specific band of 550bp in Marvi-2000, a positive control of *Lr*9 so; it did not show the presence of this gene in one hundred varieties/lines. The SSR marker Xgwm630 showed the presence of *Lr*13 in seven varieties, thirty six lines and in positive control (Egret), amplified the specific band of 120bp. Expressed sequence tag (EST) marker EF2/ER4 of *Lr*19 amplified 191bp fragment in positive control 'Agatha' and in one line (V-50) from CIMMYT. STS based maker 638F/638R for *Lr*20 did not show its presence in any wheat variety/line or Morocco. It amplified 542bp band only in positive control 'Thew'. The dominant STS marker Sr24#12 for *Lr*24 amplified a 500-bp fragment in positive control 'Lang' and four of the CIMMYT wheat lines (V-11, 12, 75, 76).

A dominant STS marker Iag95 of *Lr*26 amplified the expected 1100-bp band in seven wheat varieties, sixteen advance lines and in positive control 'PBW343'. SCAR marker $SCS421_{570}$ and STS marker mag3092 revealed absence of *Lr*28 in all wheat varieties/lines. A dominant marker $SCS421_{570}$ amplified 570bp fragment in positive control 'Sunland'. Similarly, a co-dominant marker mag3092 amplified 200bp fragment in positive control while 225bp in Morocco and in all varieties/lines. A co-dominant STS marker csLV34F/csLV34R of *Lr*34 amplified two alleles, a band of 150 bp in three of the varieties, thirty five wheat lines and in positive control but in remaining varieties/lines and in Morocco it amplified 229bp fragment (non *Lr34* carrying allele) while one of the synthetic wheat line W-5 showed both alleles. *Lr*37 linked CAPS marker VENTRIUP/LN2 amplified 259bp fragment in one of the synthetic hexaploid line W-54 and in positive control 'Trident'. Only one CIMMYT wheat genotype (V-16) and C98.6 Pavon (positive control) showed a specific band (282bp) of *Lr47* through its linked CAPS marker PS10R/PS10L2 (Fig. 1).

Table 3. Status of seventeen Pakistani wheat varieties and fifty nine wheat lines against leaf rust race FHPRN at seedling and adult plant stages with their *Lr* genes and pedigree detail. The twenty four lines were resistant to moderate (moderately resistant/susceptible) in field except V-40 (30S) and did not show the presence of studied *Lr* genes through molecular markers. The data about other *Lr* genes in Pakistani wheat varieties was taken from McIntosh *et al.* (1995); Babar *et al.* (2010); Rattu *et al.* (2010); Hussain *et al.* (2011); Ejaz *et al.* (2012) and Mustafa *et al.* (2013).

Sr. No.	Codes/Varieties/lines pedigree	Seedling Data	Field data at Fbd	Field data at Bwp	*Lr* genes of this study	Previously reported *Lr* genes
1.	BlueSilver= II53.388/AN//YT54/N10B/3/*LR*/4/B4946.A.4.18.2.1Y/Y53//3*Y50	3	30S	40S	*Lr*13	*Lr*13, 14a
2.	Pak-81= KVZ/BUHO//KAL/BB = VEE#5CM33027-F-15M-500Y-0M-76B-0Y-0PAK	;12	30S	0	*Lr*26	*Lr*10, 23, 26, 31
3.	Sh-88= PB 81/HD 2182//PB 81	34	80S	70S	-	-
4.	Pasban-90= INIA 66/A. DISTT//INIA 66/3/GEN 81	34	20MS	0	*Lr*13, *Lr*26	*Lr*10, 26, 27
5.	Auqab-2000= CROW'S'/NAC//BOW'S'	34	100S	50S	*Lr*13, *Lr*26	-
6.	BWP-97= SUSONOKOMUGI/NORIN/(SIB)BOBWHITE[2588]	4	30S	40S	*Lr*34	-
7.	BWP-2000= AVRORA/UP-301//GALLO/SUPER-X/3/(SIB)PEWEE/4/MAT(SIB)/(SIB)MAYA-74//(SIB)PEWEE[2588];	0	100S	60S	*Lr*26, *Lr*34	*Lr*10, 13, 26, 27, 31
8.	Chenab-2000= CBRD (CHUM 18/BAU)CM 92991-59M-0Y-0M-5Y-0B	0	20R	0	*Lr*13, *Lr*26	*Lr*3, 10, 13, 26, 27, 31
9.	Iqbal-2000= WL711/CROW'S'PB1954-9A-1A-0A	34	20MSS	30MS	*Lr*26	*Lr*10, 23, 27, 31
10.	As-2002= KHP/D.31708//CMH74A.370/3/CNO79/4/RL6043/*4NAC.PBD.795-23A-1A-0A	34	0	0	*Lr*26, *Lr*34	*Lr*10, 26
11.	Bhakkar-02= P-102/PIMA//F3.71/TRM/3/PVNPB-23826-D-1A-1A-1T-1T-0T	0	20R	0	*Lr*13	-
12.	Shafaq-06= LU 26/HD2179//2*INQ-91PB 28633P-2A-6A-0A	23	30R	TR	-	*Lr*10, 27, 31
13.	Seher-06= CHIL/2*STAR/4/BOW/CROW//BUC/PVN/3/--CMSS9Y00645-100Y-200M-17Y-10M-0Y	34	80S	70S	*Lr*13	*Lr*10, 13, 27
14.	Fbd-08= PBW65/2*PASTOR	34	10MRMS	20MRMS	-	*Lr*10
15.	Lasani-08= LUAN/KOH97	X	50S	0	*Lr*13	*Lr*13, 27, 31
16.	Millat-11= Chenab2000/Inqilab91	4	10MRMS	10R	-	-
17.	Punjab-11= AMSEL/ATTILA//INQ-91/PEW'S'	34	30MR	0	-	-
18.	N-7= CHAM-8 (Check 1-Syria)	23	20MS	20MRMS	*Lr*13, 26, 34	-
19.	N-23= CHAM-8 (Check 1-Syria)	3	20RMR	10R	*Lr*13	-
20.	N-34= Pak-81/01FJ14//LYP37/94-R-30	34	0	TR	*Lr*34	-
21.	N-44= V.3009/PVN	1	0	0	*Lr*26	-
22.	N-51= INQ91/2460	0	TR	0	*Lr*34	-
23.	N-52= V.3009/SH-2002	X	0	0	*Lr*26, 34	-
24.	N-55= BB/KAL//2460	34	30S	20S	*Lr*34	-
25.	N-76= BWP.2000/PARI.73	34	0	0	*Lr*34	-
26.	N-162= ERET2/TUKURU//FRET2	3	TR	0	*Lr*34	-
27.	N-163= KIRITATI/4/2*SERI.1B*2/3/KAUZ*2/BOW//KAUZ	2	0	0	*Lr*26	-
28.	N-176= NS732/HER//ARRIHANE/3/PGO/SERI//BAU	4	10R	TRMR	*Lr*26, 34	-
29.	N-282= RAWAL87X8073	X	0	TR	*Lr*13	-
30.	N-302= WATTANX8060	3	0	0	*Lr*13	-
31.	V-1= V-04017= PB96/FRONTANA//PB96	0	10R	0	*Lr*13, 26	-
32.	V-3= V-04063= SH88/PAK81//MH97	3	60S	40MSS	*Lr*13, 26, 34	-
33.	V-4= V-04 089= V87094/FSD85//V87094	34	50S	TR	*Lr*13	-
34.	V-6= V-04179= PB96/V87094//MH97	X	10MRMS	0	*Lr*13, 34	-
35.	V-7= V-03144= SH88/PAK81//MH97	34	80S	70S	*Lr*26	-
36.	V-9= V-04016= SH88/LU26//MH97	2	10R	0	*Lr*26	-
37.	V-11= V-02155= SH-88/V87094//MH97	34	TMRMS	5R	*Lr*13, 24	-

Table 3. (Cont'd.).

Sr. No.	Codes/Varieties/lines pedigree	Seedling Data	Field data at Fbd	Field data at Bwp	*Lr* genes of this study	Previously reported *Lr* genes
38.	V-12= V-02200= V87094/PB96//MH97	0	TS	5R	*Lr*24, 26	-
39.	V-15= CIMMYT= BABAX/*LR*42//BABAX*2/3KURKU	4	90S	70S	*Lr*13	-
40.	V-16=CIMMYT= BABAX/*LR*42//BABAX2*/3PAVON7S3,+*LR*47	;	5R	0	*Lr*47	-
41.	V-20= CIMMYT= FRET2/KUKUNA/FRET2	0	20R	0	*Lr*34	-
42.	V-45= CIMMYT= KIRITATI//ATTILA*2/PASTOR	34	TRMR	TR	*Lr*13	-
43.	V-48= CIMMYT= KIRITATI//PRL/2*PASTOR	X	5R	0	*Lr*34	-
44.	V-49= CIMMYT= KIRITATI//SERI/RAYON	2	0	0	*Lr*13, 34	-
45.	V-50= CIMMYT= MILAN/S87230//BABAX	0	5R	0	*Lr*19	-
46.	V-51= CIMMYT= OASIS/SKAUZ//4*BCN*2/3/PASTOR	3	10R	0	*Lr*26	-
47.	V-53= CIMMYT= PBW343/WBLLI//PANDION	4	0	0	*Lr*13	-
48.	V-55= CIMMYT= PFAU/WEAVER*2//BRAMBLING	1	60S	0	*Lr*34	-
49.	V-56= CIMMYT= PFAU/WEAVER*2//KIRTATI	0	0	0	*Lr*13, 26	-
50.	V-57= CIMMYT= PFAU/W2*EAVER*2//KIRTATI	34	5R	0	*Lr*13	-
51.	V-59= CIMMYT= THELIN//2*ATTILA*2/PASTOR	0	20RMR	0	*Lr*13	-
52.	V-62= CIMMYT= THELIN/3/2*BABAX/*LR*42//BABAX	0	10R	0	*Lr*13, 26	-
53.	V-63= CIMMYT= KAUZ/PASTOR//PBW343	34	5R	0	*Lr*13	-
54.	V-64=CIMMYT= PF4354//LD/ALD/4/2*BR12*/3/JUP//PAR214*6/FB6631/5/HP1731	34	10RMR	0	*Lr*13, 34	-
55.	V-66= RKLDSN= HAAMA-2/LAKAT-7	12	20MSS	10MSS	*Lr*13, 26, 34	-
56.	V-67= RKLDSN= SAKHA 61/MILDESSMO73/POL//AEST-BON/COM/-7C/3/2AB	0	0	0	*Lr*13, 34	-
57.	V-68= RKLDSN= KARWAN-1/MELLAL-1	23	20S	10S	*Lr*13, 34	-
58.	V-69= RKLDSN= KARWAN-1/MELLAL-1	4	10R	0	*Lr*13, 34	-
59.	V-70= RKLDSN= SOMAMA-9//SERI 82/SHUHA'S'	0	10R	0	*Lr*13, 34	-
60.	V-71= RKLDSN= SOMAMA-9//SERI 82/SHUHA'S'	34	10R	0	*Lr*13, 34	-
61.	V-72= RKLDSN= SOMAMA-9//SERI 82/SHUHA'S'	4	TS	0	*Lr*13, 34	-
62.	V-73=RKLDSN= HK 1/6/NVSR3//5/BEZ/TVR/5/CFN/BES//SU92/CI13645/3NAI60	2	TMRMS	0	*Lr*13, 34	-
63.	V-74= RKLDSN= ICW99 158-0AP-0AP-0AP-1E-0E-3E-0E	X	TS	0	*Lr*13, 34	-
64.	V-75=RKLDSN= HK 1/6/NVSR3//5/BEZ/TVR/5/CFN/BES//SU92/CI13645/3NAI60	0	40R	TR	*Lr*13, 24, 34	-
65.	V-76= RKLDSN= ICW99 158-0AP-0AP-0AP-5E-0E-2E-0E	4	60S	40S	*Lr*13, 24, 34	-
66.	V-78= RKLDSN= TCI011103	23	0	0	*Lr*13, 34	-
67.	V-79= RKLDSN= CTK/3/ATL66/CMN//TX2607-6/4/SS8/LLFN/BEZ/NAD//KZM74	;	10R	0	*Lr*13, 34	-
68.	V-80= RKLDSN= ZCL/3/PGFN//CNO67/SON64(ES86-8)/4/KA../4/ALTAY/TCI011552	3	70S	50S	*Lr*13, 26, 34	-
69.	W-14= D67.2/P66-270//AE.TAUSCHII (257)/3/OPATA	34	20MSS	30MSS	*Lr*13, 34	-
70.	W-32= ALTAR84/AE.TAUSCHII//OPATA	0	0	0	*Lr*13, 34	-
71.	W-33= CROC_1/AE.TAUSCHII (224)//OPATA	;2	0	0	*Lr*13, 34	-
72.	W-35= CHEN/AE.TAUSCHII//2*OPATA	4	0	0	*Lr*34	-
73.	W-36= ALTAR84/AE.TAUSCHII//2*OPATA	4	TR	0	*Lr*34	-
74.	W-37= CROC_1/AE.SQUARROSA (205)//KAUZ/3/SASIA	0	0	0	*Lr*26	-
75.	W-42= FILIN/IRENA/5/CNDO/R143//ENTE/MEXI_2/3/AEGILOPS SQUARROSA (TAUS)/4/WEAVER	4	0	0	*Lr*34	-
76.	W-54= PASTOR/3/ALTAR84/AEGILOPS SQUARROSA (TAUS)//OPATA	4	20RMR	10R	*Lr*37	-

*Fbd= Faisalabad *Bwp= Bahawalpur *CIMMYT= International Maize and Wheat Improvement Center *RKLDSN= Wheat lines of Faisalabad

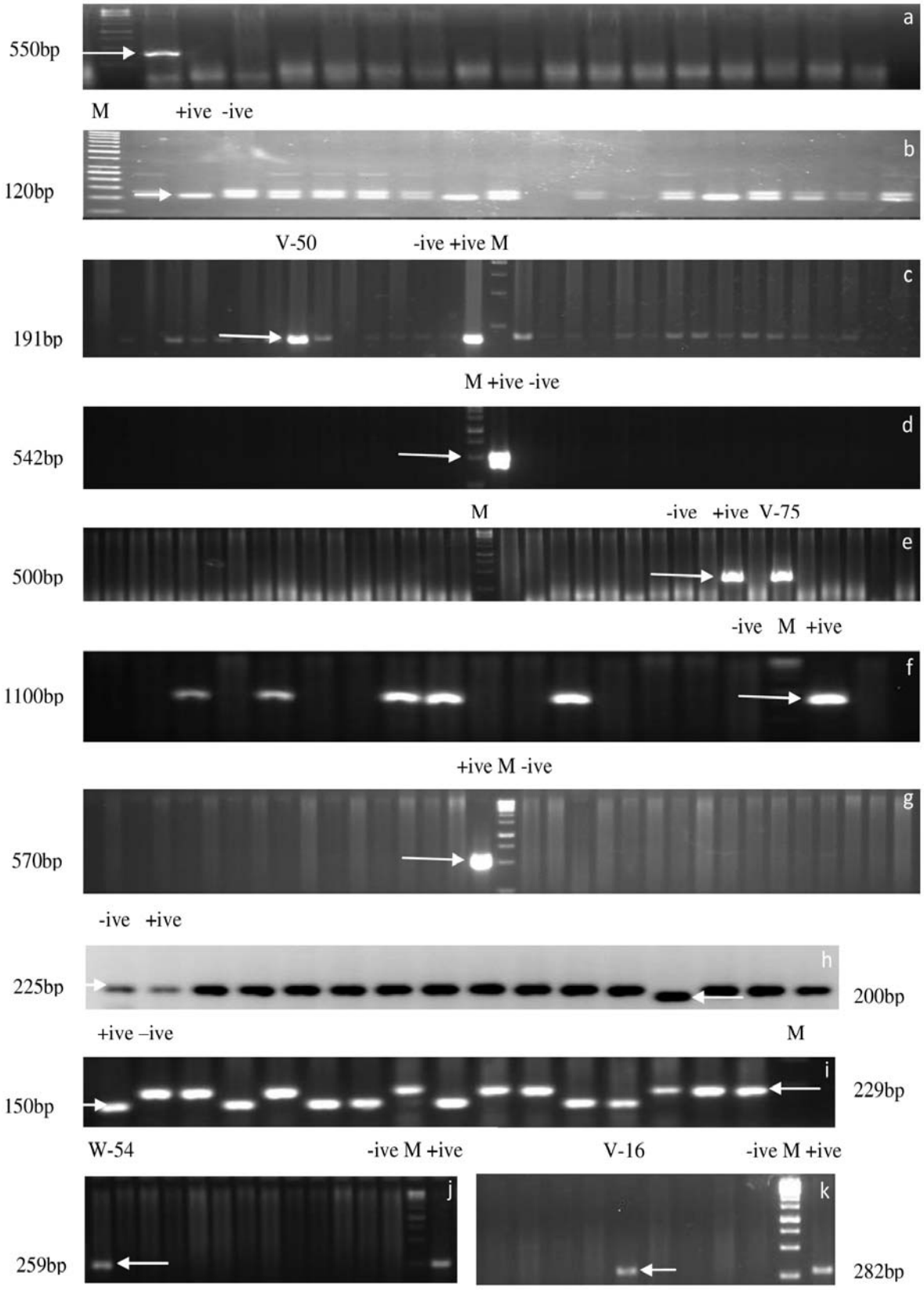

Fig. 1(a-k). Validation of molecular markers of *Lr* genes. (a) SCS_{550} of *Lr*9 (b) Xgwm630 of *Lr*13 (c) EF2/ER4 of *Lr*19 (d) 638F/638R of *Lr*20 (e) Sr24#12 of *Lr*24 (f) Iag95 of *Lr*26 (g) $SCS421_{570}$ of *Lr*28 (h) mag3092 of *Lr*28 (i) csLV34 of *Lr*34 (j) VENTRIUP/LN2 of *Lr*37 (k) PS10R/PS10L2 of *Lr*47. V-16, V-50, V-75, W-54= Codes of wheat lines; +ive= Positive controls of *Lr* genes; M= Marker (Ladder).

Discussion

Durable resistance against leaf rust can be achieved through gene stacking. Breeders stacked some of the commonly found *Lr* genes in Pakistani wheat varieties however most of these no longer confer resistance against new leaf rust pathotypes that signifies the need for new resistant gene source. The varieties with seedling resistance genes remain effective for 5-6 years generally (Singh *et al*., 2005) but with APRs these last longer. For example, a high yielding Pakistani variety Seher-06 was found susceptible during a field survey of 2011-12 against a newly emerging race FHPRN largely because of its seedling gene composition. This further highlighted the need to assess the present status of rust resistance gene(s) alone (isogenic lines) or in combination (varieties/lines) to aid in developing future breeding plans for wheat. Through linked and validated molecular markers the presence of effective gene(s)/combinations were assessed. Thus a wealth of seedling (*Lr*19, 24, 26, 47) and APR (*Lr*13, 34, 37) genes source was identified which can be used in future breeding in order to achieve durable resistance. Imbaby *et al*. (2014) also identified the same set of *Lr* genes for Egyptian wheat cultivars using same set of molecular markers.

Hitherto, seventy *Lr* genes have been identified (McIntosh *et al*., 1995; 2007; 2012) but the pathogen composition in an area determines the virulence/avirulence for the deployed genes in host. Many *Lr* genes have been defeated by rapid pathogen evolution therefore breeders are searching for new source of resistant genes. Pathogen never sleeps and it changes its nature quickly. If we analyse the history of some renowned *Lr* genes in Pakistan, a decade ago the *Lr*9 and *Lr*24 were found susceptible (Mirza *et al*., 2000). During the last decade *Lr*9 was found resistant and *Lr*24 was found susceptible (Fayaz *et al*., 2008; Rattu *et al*., 2009). Present and previous studies also agreed upon the effectiveness of *Lr*19 and *Lr*28 in the last two decades (Mirza *et al*., 2000; Fayaz *et al*., 2008; Rattu *et al*., 2009). Presently, we found a new emerging race FHPRN thriving on Seher-06 which was found highly virulent against *Lr*2b, 2c, 3, 3ka, 3bg, 10, 12, 13, 14a, 14b, 16, 17, 18, 22a, 22b, 23, 25, 26, 29, 30, 32, 33, 35, 36. Our seedling and field data revealed the effectiveness of *Lr1*, 2a, 9, 19, 24, 10+27+31, 28 and 37 against this race.

A single seedling gene with few years of effectiveness (Singh *et al*., 2005) can survive more in combination with additional genes. However, markers data collation suggested that some gene combinations in Pakistani varieties have already been defeated due to pathogen diversity e.g. *Lr*13+14a (Blue silver) (Javed *et al*., 2013) and *Lr*10+23+26+31 (Pak-81) (Hussain *et al*., 2011; Javed *et al*., 2013; Mustafa *et al*., 2013) and were also found susceptible against FHPRN. Other combinations: *Lr*10+23+26+27+31 (Auqab-2000), *Lr*3+10+13+26+27 (Iqbal-2000) and *Lr*13+27+31 (Lasani-08) previously deemed effective were no longer found effective against FHPRN (Hussain *et al*., 2011; Javed *et al*., 2013; Mustafa *et al*., 2013). Mustafa *et al*. (2013) identified *Lr*10+27+31 in Seher-06, the highly susceptible variety of the present study. This gene combination was also found ineffective in wheat variety Auqab-2000. Intriguingly, the same gene combination in Gatcher was found effective against FHPRN both at seedling and adult plant stages. This may have happened because of the presence of other gene(s). Many of the reports indicated the suppressors for leaf and stem rust resistance in genus *Triticum* (Dyck, 1987; Bai & Knott 1992) and that suppression may also be gene specific (Villareal *et al*., 1992; Ma *et al*., 1995).

The APR gene combinations are more effective than seedling gene combinations e.g. *Lr*12 and *Lr*13 increase the durability of *Lr*34 (Roelfs, 1988). Dyck *et al*. (1966) pioneered in identifying *Lr*13 and *Lr*34 in wheat variety Frontana. *Lr13* and *Lr34*, the slow rusting genes only allow the disease to spread slowly and thus reducing the damage in yield (Singh *et al*., 1991). In the present study, some of the varieties/lines were found to carry both APRs. The gene combinations of *Lr*13 and 34 together or with some of the seedling genes were found effective in the present study but somehow *Lr*26 interrupted the effectiveness of this gene combination e.g. in wheat lines V-3 (60S) and V-80 (70S). A gene combination *Lr*10+13+26+27 in Pasban-90 was found moderately susceptible in our study, though previously it was moderately resistant (Mustafa *et al*., 2013). A six genes combination *Lr*10+13+26+27+31+34 in variety Chenab-2000 was also found effective in the present as also previous studied (Mustafa *et al*., 2013). A gene combination *Lr*10+26+34 in variety As-02 was also found effective against FHPRN in field as also reported previously (Fayaz *et al*., 2008; Mustafa *et al*., 2013). *Lr*13 was found effective with *Lr*10+27 in variety Shafaq-06. Another gene combination *Lr*26+34 (N-52, N-176) was found effective in our field studies. Two more effective combinations of *Lr*13 and *Lr*34: *Lr*13+16 (Samborski & Dyk, 1982) and *Lr*16+34 (Hiebert *et al*., 2010) have been reported previously. *Lr*37 also proved moderately effective APR gene in this study which was found in one of the synthetic line W-54. A combination of *Lr*37 with *Lr*13 or 34 in combination (Kloppers & Pretorius, 1997) would achieve more durable resistance.

Pyramiding APR gene with resistant seedling genes provides an alternative effective strategy to enhance durability in wheat rust resistance (Leonard & Szabo, 2005). One of the most important seedling genes of this study was *Lr*24 which was found effective against FHPRN. Literature suggests that *Lr*24 was not effective alone but can be used in combination with other *Lr* genes (Sawhney, 1985; Kochumadhavan *et al*., 1988). In the present study, we found its effectiveness in combination with *Lr*13 and *Lr*26 e.g. *Lr*13+24 (V-11) and 24+26 (V-12) (Table 3). Some other gene combinations of *Lr*24 have also been reported previously viz., *Lr*9+24 (Long *et al*. 1994; Slikova *et al*., 2004), *Lr*24+26 (Datta *et al*., 2011), *Lr*24+26+28 (Sohail *et al*., 2014) and *Lr*21+24+34 (Gorash *et al*., 2014). Present and previous studies also endorsed the effectiveness of three other seedlings genes such as: *Lr*9, *Lr*19 and *Lr*28 (Fayaz *et al*., 2008; Rattu *et al*., 2009). Unfortunately, gene combinations of these three genes have not been assessed by breeders, though recently Sallam *et al*. (2014) revealed their effectiveness. We did not find *Lr*9 and 28 in studied wheat material while *Lr*19 was found only in CIMMYT line V-50 with effective resistance in field (5R). Datta *et al*. (2011) also reported the effectiveness of a gene combination *Lr*9+*Lr*19. Similarly, the seedling gene *Lr*47 was also found effective in one of the CIMMYT line V-16 with infection type of 5R against FHPRN in field. *Lr*47 can

also be used in combination with APRs *Lr*34 and *Lr*46 for durable resistance (Vanzetti *et al*., 2011).

In conclusion the gene combinations: *Lr13* + 34, *Lr*26 + 34, *Lr*10 + 27+31 (Gatcher), *Lr*10 + 13 + 26 + 27, *Lr*10 + *Lr*13 + 27, *Lr*10 + 13 + 26 + 27 + 31 + 34, *Lr*10 + 26 + 34, *Lr*13 + 24, *Lr*24 + 26 were found resistant; while *Lr*13 + 14a, *Lr*10 + 23 + 26 + 31, *Lr*10 + 23 + 26 + 27 + 31, *Lr*3 + 10 + 13 + 26 + 27, *Lr*13 + 27 + 31 were found susceptible to FHPRN. The wheat varieties: Pasban-90, Iqbal-2000, Chenab-2000, As-02, Bhakar-02, Shafaq-06, Fbd-08, Millat-11 and Punjab-11 proved effective resistance against FHPRN. Though, Pakistani wheat varieties are based on the genes/combinations of: *Lr*3, 10, 13, 14a, 23, 26, 27, 31 and 34 though lacking some of the more effective genes: *Lr*9, 19, 24, 28, 37 and 47. These effective genes should be incorporated in Pakistani varieties to combat FHPRN and other closely related leaf rust pathotypes thus ensuring food security in Pakistan.

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