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Full Length Article

Molecular and Phenotypic Analysis of Bread Wheat Varieties in Relation to Durable Rust Resistance

Anisa Intikhab[1], Shahid Iqbal Awan[1*], Luis A.J. Mur[2], Muhammad Sajjad Saeed[3] and Muhammad Shahzad Ahmed[4]
[1]*Department of Plant Breeding and Molecular Genetics, Faculty of Agriculture, University of Poonch Rawalakot, Azad Kashmir-Pakistan*
[2]*Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales, United Kingdom*
[3]*Vegetable Research Institute, Ayub Agricultural Research Institute, Faisalabad-Pakistan*
[4]*Rice Research Programme, Crop Sciences Institute, National Agricultural Research Centre, Islamabad-Pakistan*
[*]For correspondence: shahidiqbal@upr.edu.pk
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Abstract

Global wheat production is constantly threatened by rust diseases. Identifying resistant genes is a useful tactic to control wheat rust pathogen. Twenty-six wheat varieties were screened with twelve Simple Sequence Repeats (SSR) markers to detect rust resistant genes and the efficacy of genes was validated through field testing. The alleles *Lr32, Lr39, Lr50, SrCad* and *SrWeb* were not amplified in the varieties included in this study. The SSR markers indicated that the varieties *viz*., Chakwal-97, Bakhar-2002 and Lasani-2008 had a combination of 02 slow rusting alleles (*Lr46/Yr29* and *Yr18/Lr34*). The adult plant resistance (APR) allele *Yr17* was less prevalent and found only in BWL-97. However, Noshera-96 had a slow rusting combination of *Lr67/Yr46* and *Lr46/Yr46* alleles. The *Lr46/Yr29* identified in 50% of the varieties, *Yr18/Lr34* in 19.23%, *Lr32* in 11.54%, and multiple APR alleles in 19.32%. Their resistance was validated through a field trap nursery for 3 consecutive seasons. The slow rusting combination of *Lr46/Yr29* and *Yr18/Lr34* was comparatively more effective than *Lr67/Yr46* and *Lr46/Yr29* alleles under field conditions. The varieties Yecora-70, Lylpure-73 and Tandojam-83 showed highly susceptible phenotype. The varieties Chakwal-86, Pirsabak-2005, Fareed-2006, and Sehar-2006 showed resistant to moderately resistant phenotype at high-temperature adult-plant stage. The cluster diagram divided the varieties into two distinct clades. The clade II depicted the abundance of APR allele *Lr46/Yr29*. The varieties contain valuable sources of durable rust resistant alleles that can be exploited to deploy rust resistance in future wheat cultivars. It has been observed that the varieties approved for commercial cultivation after 1990s and onwards contain APR alleles.

Keywords: Wheat; *Triticum aestivum*; Rusts; Molecular markers; SSR

Introduction

Spring wheat is a major cereal crop of Pakistan (GOP 2020). The genetic improvement is the result of global wheat improvement efforts, but currently its production is stagnant and further enhancement is confronted by biotic (rusts, smuts and powdery mildew) (Rattu *et al.* 2011) and abiotic (terminal heat, salinity, drought, hailstorms, winds, fogs, and extreme cloud cover during cropping season) stresses (Jellis 2009). The rust diseases pose a severe biotic stress to wheat productivity caused by the members of genus *Puccinia* (Hovmøller *et al.* 2010; Zeng *et al.* 2019), it is also a major threat to Pakistani wheat (Babar *et al.* 2010). The stripe rust disease induced by a fungus *Puccinia striiformis* f. spp. *tritici* is a devastating disease of wheats grown in the temperate climate (Wellings 2011; Beddow *et al.* 2015; Ayliffe and Soerensen 2019), it reduces wheat production up to 70% (Chen 2007). Because of its epidemics inflicted during 2005 and 2012 in Pakistan, high yielding wheat varieties became susceptible (Hussain *et al.* 2015). Similarly, the *Puccinia graminis* f. spp. *Tritici* (*Pgt*) produces stem rust (Saari and Prescott 1985), a destructive disease of wheat causing 48–50% yield losses (Soko *et al.* 2018). While, *Puccinia triticina* produces leaf rust, regarded as a significant problem of wheat in different countries (Singh *et al.* 2008). Wheat leaf rust resulted in yield loss up to 74% when plants are infected at the initial stages of their growth (Herrera-Foessel *et al.* 2006).

To cope with wheat rust pathogen several methods are in practice, application of fungicides is costly, unfriendly to the environment and leads to the development of pathogen resistance (Chen 2007). The genetic resistance is considered as an economical, effective, long-term, and eco-friendly approach (Liu *et al.* 2019). It can be used as a long-term tactic to avoid crop losses. Therefore, wheat rust damage can be avoided by identifying effective rust resistant gene(s)

(Babar *et al.* 2010) and their deployment in future wheat cultivars. Gene postulation as well as molecular markers could be utilized to enhance wheat rust resistance by the detection of high-temperature adult-plant stage rust resistance genes and pyramiding them in a single cultivar (Lagudah *et al.* 2009). New evidence suggested that the durable or non-host resistance prohibit the pathogens to colonize the plants because of the molecular incompatibility among the pathogenic factors and cellular target sites of host plant. The non-host resistance is durable and remains effective for a longer duration (Ayliffe and Soerensen 2019).

Disease resistant genes provide durable resistance against pathogen and their detection by the use of molecular markers makes the selection easy. The SSR markers are widely used short tandem repeats, abundantly exist in the entire genome and more polymorphic than any other marker systems (Miah *et al.* 2013). Automation and co-dominant behaviour are additional benefits when compared to other markers (Mornkham *et al.* 2016). Several studies have been utilized to illustrate the application of phenotypic and molecular approaches for the identification of rust resistant alleles in wheat germplasm collections, but a few of the earlier reports aimed at the spring wheat varieties grown in Pakistan. In the present study, we utilized a group of 26 spring wheat varieties representing both recent and old varieties using the phenotypic and molecular data for the already reported alleles. The specific objective was to identify rust resistance genes utilized in these varieties, especially to detect genes that were not formerly reported and to report durable rust resistance sources. The other objectives included phenotyping of varieties for yellow rust resistance. The durable resistance sources and alleles should be beneficial for the development of future cultivars with effective resistance, this genetic material should be utilized immediately for the disease management.

Materials and Methods

The authenticated seed of 26 elite spring wheat varieties were obtained from Barani Agricultural Research Institute-Chakwal, National Agriculture Research Center-Islamabad, and Ayub Agricultural Research Institute-Faisalabad (Table 1).

Genotyping with SSR markers

For the extraction of genomic DNA, seed were grown in pots comprising of a mixture of leaf compost and sandy loam topsoil in equal amount. The seedlings germinated inside an incubator (Memmert-GmbH) at 24°C in the department of Plant Breeding and Molecular Genetics and later transferred to greenhouse, watered regularly, and kept at 24°C. The seedlings were harvested at 3 leaf stages and stored at -80°C (SANYO-Japan, MDF-293). The genomic DNA was isolated from seedlings by means of CTAB method of Doyle and Doyle (1987) with a little modification. The confirmation of genomic DNA was done by electrophoresis using 0.8% agarose gel, followed by quantification with the help of UV-VIS spectrophotometer (Shimadzu, UV-1201). A 10x dilution of the genomic DNA was prepared to enhance the volume and ease of mixing. The genomic DNA comprising 50 ng/μL was used for PCR.

Twelve SSR primers were utilized to screen rust resistant alleles (Table 2). The primer sequences were acquired online from MAS wheat UCDAVIS (http://maswheat.ucdavis.edu). The PCR reaction was carried out in a 10 μL reaction comprising 0.5 μL genomic DNA, pre-mix (Thermo Scientific^{TM}) 5.78 μL, double distilled water 5.70 μL and 0.4125 μL of primers both reverse and forward. The PCR reactions were carried out inside a Thermo cycler (BioRad MJ Mini) with protocols given in Table 2. The PCR products of CAPS marker S30-13 were washed, re-precipitated and restricted using BamHI. The 10x reaction buffer (2 μL) and 0.5 μL of BamHI (Thermo Scientific^{TM}) were added to re-suspended PCR product and incubated at 37^{o}C for 30 min. The PCR products were confirmed on 2.5% agarose gel (Invitrogen UltraPure^{TM}, USA). For the quantification of bands on agarose gel, 5 μL of 100bp DNA ladder (Thermo Scientific™) was used. In the end, the gels were photographed for genetic analysis inside a UVIDOC gel documentation system (UVITEC, UK).

Field testing and phenotyping

The varieties were phenotyped for reaction to yellow rust under natural conditions at University of Poonch Rawalakot (Latitude: 33°51'28.15"N, longitude: 73°45'37.55"E, elevation: 1737 m) for 03 seasons *i.e.*, 2016–2017, 2017–2018 and 2018–2019. The trials were non-replicated and planted as single row per entry (1.0 m long and 30 cm apart), with susceptible spring wheat variety 'Morocco' as an inoculum spreader. The Morocco was planted in rows perpendicular and adjacent to the rows as Wei *et al.* (2015). The natural infection permitted us to record data on stripe rust disease without artificial inoculation as Cheng *et al.* (2014). Ten flag leaves were randomly selected form each variety when the leaves of Morocco were fully infected and the grains were at the milk stage (Feekes 10.54–11.1). Disease surveys were repeated twice with 10 days interval. The infection and disease severity were scored according to the modified Cobb scale (Peterson *et al.* 1948). The disease severity of varieties was observed as single value and later averaged for each variety as Zeng *et al.* (2019).

Statistical analysis

The band size was assessed with the help of UVI-soft Image Analysis Software, Version 12.8 for Windows. Then the existence and non-existence of the DNA fragments

Table 1: List of spring wheat varieties used for phenotypic and molecular characterization of rust resistance

S. No.	Varieties	Sr. No.	Varieties	S. No.	Varieties
1.	Attila	10.	Mehran 89	19.	Pirsabak 2004
2.	Blue Silver	11.	Bakhtawar 93	20.	Pirsabak 2005
3.	Sarsabz	12.	Noshera 96	21.	Raskoo 2005
4.	PBW 343	13.	BWL 97	22.	Fareed 2006
5.	Yecora 70	14.	Chakwal 97	23.	Sehar 2006
6.	Lylpure 73	15.	BWL 2000	24.	Lasani 2008
7.	Tandojam 83	16.	Wafaq 2001	25.	Chakwal 50
8.	Punjab 85	17.	Bakhar 2002	26.	Panjab 11
9.	Chakwal 86	18.	SH 2002		

Table 2: The primers used to detect wheat rust resistance alleles in 26 spring wheat varieties

Gene	Primer	Sequence of primers (5'-3')	Reference
Lr32	*WMC43*	TAGCTCAACCACCACCCTACTG ACTTCAACATCCAAACTGACCG	Thomas *et al.* (2010)
Lr39	*GDM35*	CCTGCTCTGCCCTAGATACG ATGTGAATGTGATGCATGCA	Cox *et al.* (1994)
Lr46	*GWM259*	AGGGAAAAGACATCTTTTTTTTC CGACCGACTTCGGGTTC	Suenaga *et al.* (2003)
Lr50	*GWM382*	GTCAGATAACGCCGTCCAAT CTACGTGCACCACCATTTTG	Brown-Guedira *et al.* (2003)
Lr51	*S30-13L*	GCATCAACAAGATATTCGTTATGACC TGGCTGCTCAGAAAACTGGACC	Dvorak (1977)
Lr67	*Xcfd71-4D*	CAATAAGTAGGCCGGGACAA TGTGCCAGTTGAGTTTGCTC	Singh *et al.* (2008)
Sr28 Flanking Markers	*wPt-7004-PCR*	CTCCCACCAAAACAGCCTAC AGATGCGAATGGGCAGTTAG	Rouse *et al.* (2012)
	WMC332	CATTTACAAAGCGCATGAAGCC GAAAACTTTGGGAACAAGAGCA	Rouse *et al.* (2012)
SrCad	*FSD_RSA*	GTTTTATCTTTTTATTTC CTCCTCCCCCCA	Hiebert *et al.* (2010)
SrWeb	*GWM47 (WMS47)*	TTGCTACCATGCATGACCAT TTCACCTCGATTGAGGTCCT	Hiebert *et al.* (2010)
Yr17	*VENTRIUP-LN2*	AGGGGCTACTGACCAAGGCT TGCAGCTACAGCAGTATGTACACAAAA	Helguera *et al.* (2003)
Yr18	*csLV34*	GTTGGTTAAGACTGGTGATGG TGCTTGCTATTGCTGAATAGT	Lagudah *et al.* (2009)

visualized on gel was written in a binary data matrix in MS Excel sheet. Depending upon the effects of electrophoretic fragment spectra, the cluster diagram was prepared using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm with the help of computer software MEGA5 (Tamura *et al.* 2011).

Results

Twelve microsatellite markers related to 11 rust resistance alleles were utilized to identify the *Yr*, *Lr* and *Sr* alleles in the 26 spring wheat varieties (Table 2). The molecular markers showed that the *Lr32* allele was found in three wheat varieties Tandojam-83, Punjab-85 and Chakwal-86, it conferred a race-specific resistance (Fig. 1a). In addition, 03 alleles *Lr39, Lr50* and *Lr51* were not present in the twenty-six varieties. The *Lr46/Yr29* allele was the most prevalent, found in 13 varieties like Bakhtawar-93, Noshera-96, BWL-2000, BWL-97, Chakwal-97, Bakhar-2002, Pirsabak-2004, SH-2002, Pirsabak-2005, Punjab-11, Raskoo-2005, Sehar-2006, and Lasani-2008. The slow rusting allele *Lr67/Yr46* was identified in Noshera-96 using the molecular marker test.

The *WMC332* marker amplified *Sr28* allele in three varieties *viz.*, BWL-2000, SH-2002 and Raskoo-2005 while that of *wPt-7004-PCR* marker was found in Pirsabak-2005 (Fig. 1b and c). The SSR markers also indicated that stem rust resistance alleles *SrCad* and *SrWeb* were absent among the tested varieties (Fig. 1d). The presence of *Yr17* allele was not high in the tested varieties; however, it was identified in BWL-97. Similarly, *Yr18/Lr34* was present in Attila, Sarsabz, Chakwal-97, Bakhar-2002, Fareed-2006, and Lasani-2008 (Fig. 1e). However, *Yr18/Lr34* can present low infection type in combination with all-stage resistance alleles. The allele has conferred leaf rust resistance in excess of 50 years and is extensively used in wheat breeding (McIntosh *et al.* 1995; Krattinger *et al.* 2009; Wei *et al.* 2015).

The molecular data of rust resistance alleles were validated by field testing for 03 consecutive Rabi seasons (Table 3). The varieties Chakwal-86, Pirsabak-2005, Fareed-2006, Sehar-2006 showed moderate resistance under field tests. These findings suggested the existence of at least 01 adult-plant resistance (APR) allele in the varieties.

Table 3: Rust resistance alleles observed in spring wheat varieties *via* SSR markers and their 03 years field response against stripe rust

S. No	Varieties	Alleles Identified through SSR Markers	*Yr* Response		
			2016-2017	2017-2018	2018-2019
1	Attila	*Yr18/Lr34*	40MS	30MS	50MS
2	Blue Silver	-	60MSS	20MSS	60MSS
3	Sarsabz	*Yr18/Lr34*	70MS	60MS	80MS
4	PBW-343	-	40MSS	30MSS	40MSS
5	Yecora-70	-	40S	60S	80S
6	Lylpure-73	-	50S	90S	60S
7	Tandojam-83	*Lr32*	70S	90S	60S
8	Punjab-85	*Lr32*	70MSS	60MSS	70MSS
9	Chakwal-86	*Lr32*	30RMR	40RMR	50RMR
10	Mehran-89	-	30MSS	90MS	60MS
11	Bakhtawar-93	*Lr46/Yr29*	80MS	10MS	70MS
12	Noshera-96	*Lr46/Yr29, Lr67/Yr46*	60MS	80MS	70MS
13	BWL-97	*Lr46/Yr29, Yr17*	40MS	60MS	40MS
14	Chakwal-97	*Lr46/Yr29, Yr18/Lr34*	5MSS	10MSS	60MSS
15	BWL-2000	*Lr46/Yr29, Sr28* (*WMC332*)	20MS	5MS	20MS
16	Wafaq-2001	-	30MSS	40MSS	80MSS
17	Bakhar-2002	*Lr46/Yr29, Yr18/Lr34*	70MS	20MS	50MS
18	SH-2002	*Lr46/Yr29, Sr28* (*WMC332*)	80MSS	60MSS	80MSS
19	Pirsabak-2004	*Lr46/Yr29*	20MSS	40MSS	50MSS
20	Pirsabak-2005	*Lr46/Yr29, Sr28* (*wPt-7004-PCR*)	5MR	10MR	40MR
21	Raskoo-2005	*Lr46/Yr29, Sr28* (*WMC332*)	30MSS	40MSS	60MSS
22	Fareed-2006	*Yr18/Lr34*	10RMR	5RMR	30MR
23	Sehar-2006	*Lr46/Yr29*	20MR	20MR	60MR
24	Lasani-2008	*Lr46/Yr29, Yr18/Lr34*	10MS	70MS	60MS
25	Chakwal-50	-	40MSS	60MSS	70MSS
26	Panjab-11	*Lr46/Yr29*	50MSS	90MSS	60MSS

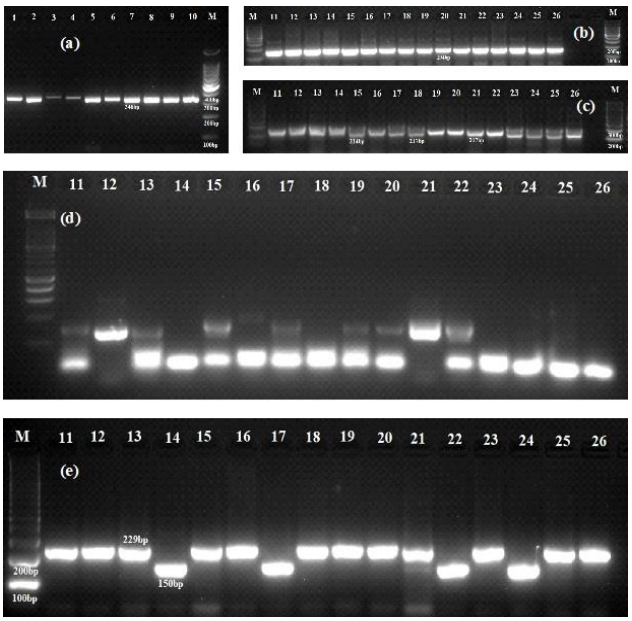

Fig. 1: The PCR products obtained from allele specific markers: *Lr32* (a), *Sr28* (**b**, **c**), *SrWeb* (**d**) and *Yr18/Lr34* (**e**) in the 26-spring wheat varieties

Molecular markers showed that resistance conferred by Pirsabak-2005, Fareed-2006 and Sehar-2006 was conditioned by APR alleles, but Chakwal-86 did not contain currently used APR markers, suggesting that the slow rusting in this variety was conditioned by other APR allele(s). While Yecora-70 (80S), Lylpure-73 (90S) and Tandojam-83 (90S) showed highly susceptible phenotype (IT) depicting the absence of effective Yr alleles against prevalent pathotypes at the adult stage. Most of the varieties contain slow rusting alleles produced moderately susceptible response.

The cluster diagram based on molecular evidence for wheat rust resistance genes could be divided into 02 distinct clades, I and II (Fig. 2). The clade I could be sub-divided into 03 distinct clusters, the sub-cluster 1 comprised of the varieties in which all the alleles under study were absent. The varieties in sub-cluster 2 had *Yr18/Lr34*, a slow rusting allele while the varieties of sub-cluster 3 had a seedling resistance allele (*Lr32*). The clade II constituted the most important group of varieties showing preponderance of *Lr46/Yr29* allele. The cluster 2 could be categorized into 05 sub-clusters, the sub-cluster 1 represented Pirsabak-2005 including slow rusting allele *Lr46/Yr29* and an unconfirmed *Sr28* allele, amplified by primer *wPt-7004-PCR*, the amplification of another flanking marker was not observed. The resistance conferred by varieties in sub-cluster 2 was furnished by two durable rust resistance alleles *Lr46/Yr29* and *Yr18/Lr34*; it was the most important group of varieties to be utilized for the incorporation of durable rust resistance in future wheat varieties. The sub-cluster 3 stemmed into a single variety Noshera-96 containing another important combination of durable rust resistance comprised of the *Lr46/Yr29* and *Lr67/Yr46* alleles. The sub-cluster 4 characterized three varieties including *Lr46/Yr29* and *Sr28* alleles, the *Sr28* was amplified only in primer *WMC332*,

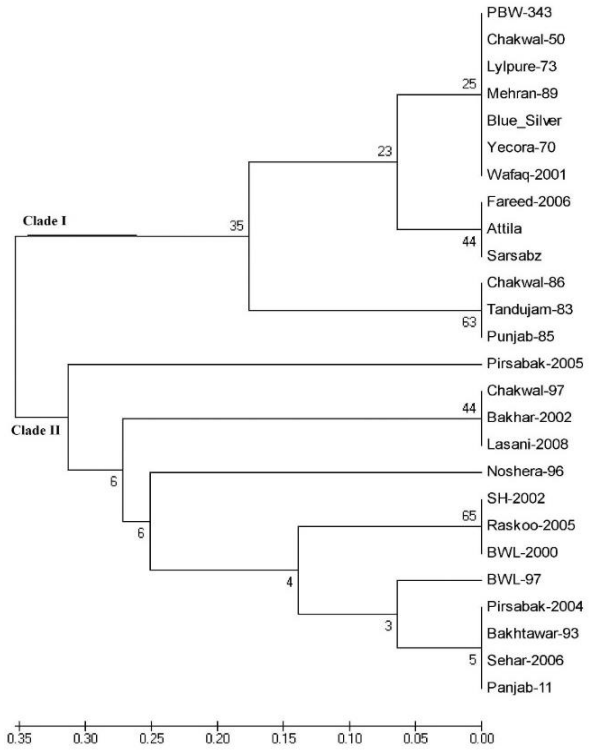

Fig. 2: Tree diagram constructed for 26 spring wheat varieties using presence/absence matrix obtained from 11 SSR primers

while the PCR amplification of other flanking marker was missing. The sub-cluster 5 characterized the variety BWL-97 including non-host durable resistance alleles *Lr46/Yr29* and *Yr17*, could be used as a source of durable rust resistance alleles.

Discussion

A set of 26 wheat varieties was screened for resistance against wheat stem rust, stripe rust, and leaf rust diseases using 12 SSR markers. The marker *WMC-43* produces a 346bp fragment linked to the occurrence of *Lr32* allele (Thomas *et al.* 2010), only Tandojam-83, Punjab-85 and Chakwal-86 amplified the correct band (Fig. 1a.). Thomas *et al.* (2010) reported that the influence of *Lr32* includes: test weight, yield increase, straw strength, grain size and hardness. The allele has shown worldwide resistance, but virulence has been spotted in South Africa (Singh 1991; Pretorius and Bender 2010). The primer *GDM-35* was used to identify *Lr39* allele among the tested varieties. The 185bp band indicates the presence of *Lr39* gene (Cox *et al.* 1994) but the PCR products ranged from 206 to 255bp indicating the absence of allele.

The *GWM259* amplifies a PCR fragment of 100–120 bp indicating the presence of slow rusting allele *Lr46/Yr29* (Suenaga *et al.* 2003). This allele was the most prevalent reported in 13 varieties constituting 50% of the varieties. Similarly, the primer *GWM-382* was used for the identification of *Lr50*, it amplifies 139bp diagnostic allele (Brown-Guedira *et al.* 2003). But the alleles ranging from 623bp to 768pb were amplified indicating absence of allele. A combination *Lr50* and other alleles could be used as a leaf rust management strategy (Brown-Guedira *et al.* 2003). The *Lr51* was amplified by the CAPS marker S30-13L primer, amplifies 672-bp and 111-bp alleles (Dvorak 1977). The *Lr39*, *Lr50* and *Lr51* are useful all-stage resistance alleles, their virulence has been observed in some parts of the world, but they are effective in combination with APR alleles (Raupp *et al.* 2001; Huang and Gill 2001). The PCR products of correct size for *Lr39, Lr50* and *Lr51* alleles had not been observed in the varieties suggesting the absence of alleles. After PCR amplification the *Lr67/Yr46* produces 214 bp product (Singh *et al.* 2008). Since this APR allele was present in Noshera-96 it could be utilized as a source for future breeding.

The flanking markers, *wPt-7004-PCR* and *WMC332*, were utilized to identify the existence of the *Sr28* allele (Fig. 1b and c). Because of the partial resistance conferred by *Sr28* allele, it is recommended as a part of gene pyramiding strategy. The PCR products of 220, 217 and 214bp for marker *WMC332* linked to the *Sr28* allele were produced. While a 194bp band associated with *wPt-7004-PCR* indicated the existence of *Sr28* allele (Rouse *et al.* 2012). The stem rust resistance conferred by *Sr28* allele shows a low IT at seedling stage (Jin *et al.* 2007). The *WMC332* allele was amplified in BWL-2000, SH-2002, and Raskoo-2005. While, *wPt-7004-PCR* allele was present in Pirsabak-2005. Since both the alleles amplified by flanking markers were not present in any of the varieties, therefore they are not recommended to be used as a source of *Sr28* allele.

The *SrCad* allele produces low infection type against stem rust resistance when combined with *Yr18*/*Lr34* allele, otherwise it offers moderate resistance against the *Ug99* and related stem rust races. While, the *GWM47* marker mapped near *Sr9* on chromosome 2BL, is utilized to identify *SrWeb* allele that presents resistance against *Ug99*. The allele size for *WMS47* is 207 bp (Hiebert *et al.* 2010). But the tested varieties yielded a DNA band of 275 bp suggesting the absence of allele (Fig. 1d).

The primers *VENTRIUP-LN2* and *csLV34* were utilized to identify the slow rusting alleles *Yr17* and *Yr18/Lr34* respectively. The slow rusting allele *Yr17* was less prevalent among the varieties found only in BWL-97. More varieties must be explored to identify the sources for this allele for future breeding. Similarly, the *csLV34* amplifies a 150 bp positive allele and a 229 bp product which is a null allele (Lagudah *et al.* 2009; Awan *et al.* 2017). The allele was of moderate occurrence observed in Bakhar-2002, Attila, Sarsabz, Fareed-2006, Chakwal-97 and Lasani-2008 (Fig. 1e).

Identification of stripe rust resistance alleles and hybridization of resistant lines is an efficient technique to reduce rust susceptibility in wheat (Li *et al.* 2006). The objective of wheat breeding targeting rust resistance is to obtain durable genetic resistance, found in adult-plant stage

slow-rusting alleles (Singh *et al.* 2008; Liu *et al.* 2020). When wheat cultivars with all-stage rust resistance are cultivated over a large area, selection pressure is exerted on the pathogen to mutate and produce new races to the break the resistance of host plant (Khan *et al.* 2011; Brar and Kutcher 2016). Gene stacking or merging many resistance alleles into a single cultivar can be used to produce durable resistance so that the pathogen can not overcome it easily (Ali *et al.* 2018). Therefore, a continuous search for new alleles for rust resistance is needed (Jiang *et al.* 1994; Abebele and Admasu 2020).

Half of the varieties indicated the incidence of *Lr46/Yr29* allele offering high-temperature resistance. The latency period of the infected plant increases when it carries *Lr46/Yr29* allele. In addition, it causes an early abortion of fungal colonies and produces a low IT but the resistance conferred is reduced in effect when compared to *Yr18/Lr34* allele (Martinez *et al.* 2001). Five varieties constituting 19.23% showed slow rusting allele *Lr46/Yr29* in combination with other durable resistance alleles (Table 3). The virulence against *Yr17* and *Yr18/Lr37* has been reported (Helguera *et al.* 2003; Sufyan *et al.* 2021) they confer moderate resistance against different physiological races and are used in combination with other rust resistance alleles. The allele *Yr17* was found in BWL-97 in combination with *Lr46/Yr29*. The *Yr18/Lr34* allele provides APR against wheat stripe/leaf rust disease and powdery mildew (*Pm38*) as reported by Juliana *et al.* (2015). Its genetic mechanism is specified as an ABC transporter (Martinez *et al.* 2001). The *Yr18/Lr37* in combination with *Lr46/Yr29* allele was present in varieties Chakwal-97, Bakhar-2002 and Lasani-2008. The *Lr67/Yr46* is an APR allele that provides a lesser level of leaf rust protection compared to *Yr18/Lr34* allele (Lagudah *et al.* 2009). It has been reported that *Lr67/Yr46* also confers APR to stem rust and powdery mildew in wheat (Herrera-Foessel *et al.* 2014; Esse *et al.* 2020). The Noshera-96 having allele *Lr67/Yr46* in combination with *Lr46/Yr29* could be a valuable source of APR for future cultivars.

Most of the varieties showed moderately susceptible phenotype at adult plant stage under Rawalakot conditions but the varieties *viz.*, Chakwal-86, Pirsabak-2005, Fareed-2006 and Sehar-2006 were moderately resistant to the prevailing stripe rust races of Rawalakot. Fayyaz *et al.* (2019) also indicated moderate resistance in these varieties. Our molecular test indicated the prevalence of horizontal resistance alleles, similarly field test depicted a low infection types in these varieties. The complete susceptibility of Yecora-70, Lylpure-73 and Tandojam-83 (Table 3) was supported by Singh and Rajaram (1992) who reported susceptibility in Yecora-70, while Afzal *et al.* (2010) described Tandojam-83 and Lylpure-73 as susceptible.

The APR genes were effective in producing low infection type in combination with other minor genes, showing moderately susceptible reactions. The *Yr18/Lr34* showed lesser susceptibility compared to *Lr46/Yr29* allele, as indicated by Martinez *et al.* (2001). The combination of durable resistance alleles like *Lr46/Yr29, Yr18/Lr34* produced low infection type in varieties Lasani-08, Bakhar-2002 and Chakwal-97. Similarly, the slow rusting combination of *Lr46/Yr29* & *Lr67/Yr46* in Noshera-96 and *Lr46/Yr29* & *Yr17* in BWL-97 was effective in producing low IT.

Cluster analysis is the name given to a set of techniques which indicates whether data can be grouped into categories based on the similarities or differences (McIntosh *et al.* 2010; Shengping and Berdine 2018). Cluster analysis based on molecular data is considered useful in identifying genetic diversity and similarities among wheat cultivars (Sobia *et al.* 2010). The cluster diagram based on presence and absence of alleles indicated two distinct clades (Fig. 2). The clade 1 could be divided into three sub-clusters while clade 2 could be categorized into five sub-clusters. Both the clusters contained equal number of varieties; the varieties of cluster II were comparatively more valuable due to the presence of durable rust resistance alleles.

Conclusion

In summary, using the combination of gene-tagging markers, we detected several alleles for resistance to rust diseases in the panel of 26 spring wheat varieties mainly used in Pakistan and identified them in individual entries. Most of the alleles produced non-race-specific APR. The durable resistance sources will enrich the resources of wheat rust resistance. The effectiveness of each previously reported alleles was assessed, and the cumulative effect of *Yr18/Lr34, Lr46/Yr29, Yr17 and Lr67/Yr46* genes was found effective in reducing disease severity. Accumulating multiple rust resistance alleles produced low infection type. However, more effective alleles conferring different types of resistance should be selected in different combinations for incorporation into new wheat cultivars. The identified sources of resistance like Chakwal-97, Bakhar-2002, Lasani-2008, BWL-97 and Noshera-96 should be useful in marker-assisted-selection for incorporating combinations of alleles. Since most of the alleles identified in the present study are present in adapted varieties, their deployment into new cultivars will be relatively easy.

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Author Contribution

Shahid Iqbal Awan designed the experiment. Anisa Intikhab performed field experiments and wrote the initial manuscript. Luis AJ Mur provided input on data analysis. Muhammad Sajjad Saeed provided input on planning of field experiments. Muhammad Shahzad Ahmed curated the molecular data and provided input on collection of field data. All authors contributed to the final draft.

Conflict of Interest

The authors declare that they have no conflict of interest.

Data Availability

Data are available from the authors on request.

Ethics Approval

The research work was conducted after approval from the Human & Animal Ethics Committee of the University and no humans and animals were investigated.

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References

Abebele GM, MA Admasu (2020). Evaluation of bread wheat genotypes for adult plant resistance to stem rust. *Food Sci Qual Manage* 103:13–18

Afzal SN, MI Haque, A Rauf, I Ahmad, SS Firdous (2010). Vulnerability of Pakistani wheat (*Triticum aestivum* L.) varieties against stripe rust under rain-fed climate of the Northern Punjab and NWFP. *Pak J Bot* 42:2029–2042

Ali Y, MA Khan, M Atiq, M Hussain (2018). Novel gene pyramiding to combat rusts in global wheat varieties against prevalent virulence: A review. *Sarhad J Agric* 34:797–810

Awan SI, SD Ahmad, LAJ Mur, MS Ahmed (2017). Marker-assisted selection for durable rust resistance in a widely adopted wheat cultivar "Inqilab-91". *Intl J Agric Biol* 19:1319–1324

Ayliffe M, C Soerensen (2019). Plant nonhost resistance: Paradigms and new environments. *Curr Opin Plant Biol* 50:104–113

Babar M, AF Mashhadi, A Mehvish, AN Zahra, R Waheed, A Hasnain, S Rahman, N Hussain, M Ali, I Khaliq, A Aziz (2010). Identification of rust resistance genes *Lr10* and *Sr9a* in Pakistani wheat germplasm using PCR based molecular markers. *Afr J Biotechnol* 9:1144–1150

Beddow JM, PG Pardey, Y Chai, TM Hurley, DJ Kriticos, HJ Braun, RF Park, WS Cuddy, T Yonow (2015). Research investment implications of shifts in the global geography of wheat stripe rust. *Nat Plants* 1; Article 15132

Brar GS, HR Kutcher (2016). Race characterization of *Puccinia striiformis* f. spp. *tritici*, the cause of wheat stripe rust, in Saskatchewan and Southern Alberta, Canada and virulence comparison with races from the United States. *Plant Dis* 100:1744–1753

Brown-Guedira GL, S Singh, AK Fritz (2003). Performance and mapping of leaf rust resistance transferred to wheat from *Triticum timopheevii* sspp. *armeniacum*. *Phytopathology* 93:784–789

Chen XM (2007). Challenges and solutions for stripe rust control in the United States. *Aust J Agric Res* 58:648–655

Cheng P, LS Xu, MN Wang, DR See, XM Chen (2014). Molecular mapping of genes *Yr64* and *Yr65* for stripe rust resistance in hexaploid derivatives of durum wheat accessions PI 331260 and PI 480016. *Theor Appl Genet* 127:2267–2277

Cox TS, WJ Raupp, BS Gill (1994). Leaf rust resistance genes Lr41, Lr42, and Lr43 transferred from *Triticum tauschii* to common wheat. *Crop Sci* 34:339–343

Doyle JJ, JL Doyle (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15

Dvorak J (1977). Transfer of leaf rust resistance from *Aegilops speltoides* to *Triticum aestivum*. *Can J Genet Cytol* 19:133–141

Esse HPV, TL Reuber, DVD Does (2020). Genetic modification to improve disease resistance in crops. *New Phytol* 225:70–86

Fayyaz M, A Munir, KA Khanzada, S Asad (2019). *Report on screening of wheats against leaf, yellow, stem rusts, spot blotch and powdery mildew*, pp:1–150. Crop Diseases Research Institute, Department of Plant & Environmental Protection, National Agricultural Research Centre, Islamabad, Pakistan

GOP (2020). *Pakistan Economic Survey*, p:18. Pakistan Bureau of Statistics, Government of Pakistan, Finance Division, Islamabad, Pakistan

Helguera M, IA Khan, J Kolmer, D Lijavetzky, L Zhong-Qi, J Dubcovsky (2003). PCR assays for the *Lr37-Yr17-Sr38* cluster of rust resistance genes and their use to develop isogenic hard red spring wheat lines. *Crop Sci* 43:1839–1847

Herrera-Foessel SA, RP Singh, M Lillemo, J Huerta-Espino, S Bhavani, S Singh, C Lan, V Calvo-Salazar, ES Lagudah (2014). *Lr67/Yr46* confers adult plant resistance to stem rust and powdery mildew in wheat. *Theor Appl Genet* 127:781–789

Herrera-Foessel SA, RP Singh, H Espino, J Crossa, J Yuen, A Djurle (2006). Effect of leaf rust on grain yield and yield traits of durum wheats with race-specific and slow rusting resistance to leaf rust. *Plant Dis* 90:1065–1072

Hiebert CW, TG Fetch, T Zegeye, JB Thomas, DJ Somers, DG Humphreys, BD Mccallum, S Cloutier, D Singh, DR Knott (2010). Genetics and mapping of seedling resistance to *Ug99* stem rust in Canadian wheat cultivars 'Peace' and 'AC Cadillac'. *Theor Appl Genet* 121:65–69

Hovmøller MS, W Stephanie, FJ Annemarie (2010). Escalating threat of wheat rust. *Science* 329:369–370

Huang L, BS Gill (2001). An RGA-like marker detects all known *Lr21* leaf rust resistance gene family members in *Aegilops tauschii* and wheat. *Theor Appl Genet* 103:1007–1013

Hussain M, MA Khan, M Hussain, N Javed, I Khaliq (2015). Monitoring of rust virulence pattern through avirulence/virulence formula. *Arch Phytopathol Plant Prot* 48:421–433

Jellis GJ (2009). Crop plant resistance to biotic and abiotic factors: Combating the pressures on production systems in a changing world. crop plant resistance to biotic and abiotic factors: Current potential and future demands. *In: Proceedings of 3rd International Symposium on Plant Protection and Plant Health in Europe*, pp:15–20. Berlin-Dahlem, Germany

Jiang J, B Friebe, BS Gill (1994). Recent advances in alien gene transfer in wheat. *Euphytica* 73:199–212

Jin Y, RP Singh, RW Ward, R Wanyera, M Kinyua, P Njau, T Fetch, ZA Pretorius, A Yahyaoui (2007). Characterization of seedling infection types and adult plant infection responses of monogenic *Sr* gene lines to race TTKS of *Puccinia graminis* f. spp. *tritici*. *Plant Dis* 91:1096–1099

Juliana P, JE Rutkoski, JA Poland, RP Singh, S Murugasamy, S Natesan, H Barbier, ME Sorrells (2015). Genome-wide association mapping for leaf tip necrosis and pseudo-black chaff in relation to durable rust resistance in wheat. *Plant Genome* 8; Article 0002

Khan MA, SM Zargar, RG Saini (2011). A novel hypersensitive stripe rust (*Puccinia striiformis* Westend f. spp. *tritici*) resistance gene in bread wheat cultivar cook effective in India. *J Phytol* 3; Article 81014137

Krattinger SG, ES Lagudah, W Spielmeyer, RP Singh, J Huerta-Espino, H McFadden, E Bossolini, LL Selter, B Keller (2009). A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. *Science* 323:1360–1363

Lagudah ES, SG Krattinger, S Herrera-Foessel, RP Singh, J Huerta-Espino, W Spielmeyer, G Brown-Guedira, IL Selter, B Keller (2009). Gene-specific markers for the wheat gene *Lr34/Yr18/Pm38* which confers resistance to multiple fungal pathogens. *Theor Appl Genet* 119:889–898

Li ZF, XC Xia, XC Zhou, YC Niu, ZH He, Y Zhang, GQ Li, AM Wan, DS Wang, XM Chen, QL Lu, RP Singh (2006). Seedling and slow rusting resistance to stripe rust in Chinese common wheats. *Plant Dis* 90:1302–1312

Liu L, M Wang, Z Zhang, DR See, X Chen (2020). Identification of stripe rust resistance loci in U.S. spring wheat cultivars and breeding lines using genome-wide association mapping and *Yr* gene markers. *Plant Dis* 104:2181–2192

Liu L, M Wang, J Feng, DR See, X Chen (2019). Whole-genome mapping of stripe rust resistance quantitative trait loci and race specificity related to resistance reduction in winter wheat cultivar Eltan. *Phytopathology* 109:1226–1235

Martinez F, RE Niks, RP Singh, D Rubiales (2001). Characterization of *Lr46*, a gene conferring partial resistance to wheat leaf rusts. *Hereditas* 135:111–114

McIntosh AM, M Sharpe, SM Lawrie (2010). Research methods, statistics and evidence-based practice. *In: Companion to Psychiatric Studies*, 8th Edition, pp:157–198. Johnstone EC, DC Owens, SM Lawri, AM McIntosh, M Sharpe (Eds). Elsevier Ltd, Amsterdam, The Netherlands

Mcintosh RA, CR Wellings, RF Park (1995). *Wheat Rusts: An Atlas of Resistance Genes*. Kluwer Academic Publishers, Dordrecht, The Netherlands

Miah G, MY Rafii, MR Ismail, AB Puteh, HA Rahim, K Nurul-Islam, MA Latif (2013). A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. *Intl J Mol Sci* 14:22499–22528

Mornkham T, PP Wangsomnuk, XC Mo, FO Francisco, LZ Gao, H Kurzweil (2016). Development and characterization of novel EST-SSR markers and their application for genetic diversity analysis of Jerusalem artichoke (*Helianthus tuberosus* L.). *Genet Mol Res* 15; Article 15048857

Peterson RF, AB Campbell, AE Hannah (1948). A diagrammatic scale for estimating rust intensity on leaves and stems of cereals. *Can J Res* 60:496–500

Pretorius ZA, CM Bender (2010). First report of virulence for the wheat leaf rust (*Puccinia triticina*) resistance gene *Lr32* in South Africa. *Plant Dis* 94:381-381

Rattu AR, S Asad, M Fayyaz, M Zakria, S Iftikhar, Y Ahmad (2011). Status of foliar diseases of wheat in Punjab, Pakistan. *Mycopathology* 9:39–42

Raupp WJ, S Sukhwinder, GL Brown-Guedira, BS Gill (2001). Cytogenetic and molecular mapping of the leaf rust resistance gene *Lr39* in wheat. *Theor Appl Genet* 102:347–352

Rouse MN, IC Nava, S Chao, JA Anderson, Y Jin (2012). Identification of markers linked to the race *Ug99* effective stem rust resistance gene *Sr28* in wheat (*Triticum aestivum* L.). *Theor Appl Genet* 125:877–885

Saari EE, JM Prescott (1985). World distribution in relation to economic loss. *In: The Cereal Rusts Volume II: Diseases, Distribution, Epidemiology and Control*, pp:259–289. AP Roelfs, WR Bushnell (Eds). Academic Press, Orlando, Florida, USA

Shengping Y, G Berdine (2018). Cluster analysis. *Southwest Resp Crit Care Chron* 6:37–40

Singh RP (1991). Pathogenicity variations of *Puccinia recondita* f. spp. *tritici* and *P. graminis* f. spp. *tritici* in wheat-growing areas of Mexico during 1988 and 1989. *Plant Dis* 75:790–794

Singh RP, S Rajaram (1992). Genetics of adult-plant resistance of leaf rust in 'Frontana' and three CIMMYT wheats. *Genome* 35:24–31

Singh RP, PD Hodson, J Huerta-Espino, Y Jin, P Njau, R Wanyera, SA Herrera-Foessel, RW Ward (2008). Will stem rust destroy the world's wheat crop? *Adv Agron* 98:271–309

Sobia T, A Muhammad, X Chen (2010). Evaluation of Pakistani wheat germplasms for stripe rust resistance using molecular markers. *Sci Chin Life Sci* 53:1123–1134

Soko T, CM Bender, R Prins, ZA Pretorius (2018). Yield loss associated with different levels of stem rust resistance in bread wheat. *Plant Dis* 102:2531–2538

Suenaga K, RP Singh, J Huerta-Espino, HM William (2003). Microsatellite markers for genes *Lr34*/*Yr18* and other quantitative trait loci for rust and stripe rust resistance in bread wheat. *Phytopathology* 93:881–890

Sufyan M, JI Mirza, M Saeed, M Fayyaz, KA Khanzada, AR Rattu, MQ Kakar (2021). Yellow rust races found in 2018-2019 collection from wheat growing areas of Pakistan. *Pure Appl Biol* 10:1063–1069

Tamura K, D Peterson, N Peterson, G Stecher, M Nei, S Kumar (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739

Thomas J, S Nilmalgoda, C Hiebert, B Mccallum, G Humphreys, R Depauw (2010). Genetic markers and leaf rust resistance of the wheat gene *Lr32*. *Crop Sci* 50:2310–2317

Wei X, H Zhang, D Du, W Yang, D Liu (2015). Evaluation of wheat leaf rust resistance genes in 10 wheat genotypes. *J Plant Dis Prot* 122:91–99

Wellings CR (2011). Global status of stripe rust: A review of historical and current threats. *Euphytica* 179:129–141

Zeng Q, J Wu, S Liu, S Huang, Q Wang, J Mu, S Yu, D Han, Z Kang (2019). A major QTL co-localized on chromosome 6BL and its epistatic interaction for enhanced wheat stripe rust resistance. *Theor Appl Genet* 132:1409–1424