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Application of Phenotypic and Molecular Markers to Combine Genes for Durable Resistance against Rust Virulences and High Yield Potential in Wheat

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Abstract

In order to combine genes for enhancing rust resistance and high yield potential in wheat, parent lines were selected for hybridization on the basis of slow rusting history and phenotypic characters for durable resistance. The hybridized germplasm was advanced in filial generations from F_1 to F_5. Total 750 head rows were planted in F_6 from selected heads among F_5 generation. From 750 single head rows planted in Kaghan, 345 lines were selected on the basis of agronomic traits and rust resistance; two hundred and twenty lines were selected for high yield and rust resistance performance and evaluated for the presence of durable rust resistant genes with molecular markers. It was confirmed that the lines showing durable rust resistance possessed *Lr34/Yr18*, *Lr46/Yr29* and *Sr2/Yr30* genes in combination or individuals from these lines. The results indicated that the most prominent lines i.e., V-11211, V-11212, V-11218, V-11227, V-11262, V-11288, V-11296, V-11304, V-11308, V-11319, V-11338, V-11353, V-11365 and V-11396 showed the combination of three designated slow rusting genes. These lines were high yielding with better resistance than all existing approved wheat varieties of the country. None of these lines had complete resistance, but were of slow rusting type and were suitable for commercial cultivation. These results will be useful for wheat breeders and pathologists of the country in planning of future hybridization program. © 2015 Friends Science Publishers

Keywords: Breeding; Resistance; Slow rusting genes; *Triticuma estivum*; Leaf and stripe rust

Introduction

Wheat crop is hit by many biotic and abiotic maladies which engender to reduce its production (Jellis, 2009). Leaf rust caused by *Puccinia recondita* Rob. exdesm. f. sp. *Tritici* also called as brown rust, stripe rust caused by *Puccinia striiformis* Westend f. sp. *tritici* also called as yellow rust and stem rust caused by *Puccinia graminis* Pers. f. sp. *tritici* also called as black rust and smuts, bunts and insects particularly aphids act as biotic stresses (Hussain *et al*., 2006) while, terminal heat, drought, salinity, winds, hailstorms, fogs and excessive cloudy weather during crop season are the salient abiotic stresses (Hussain *et al*., 2011). Rusts being important worldwide are known for their ability to mutate and multiply rapidly and to use their air-borne dispersal mechanism from one field to another and even over longer distances. Rusts are currently the most important diseases of wheat worldwide, which threaten global food security (Hovmøller *et al*., 2010). Major wheat growing areas of the world are facing repeated severe yellow rust epidemics since 2000, when two highly aggressive and high temperature tolerant *Pst* strains appeared (Hovmøller *et al*., 2008). In Pakistan, rusts have been a constant threat to sustainable wheat production, although no severe leaf rust epidemic occurred after 1978 in the country, mainly because of release of rust resistant varieties, however, except, Inqilab-91, the average life of a variety happened to be around 5 years (Hussain *et al*., 1999). The reason for the early collapse of varieties is linked to the evolution of new rust races, rendering resistance in the varieties ineffective based on major genes. The latest and current trend of genetic resistance in wheat is "the resistance based on the additive effects of minor genes accumulation" (Singh *et al*., 1998). The durable resistance to leaf and stripe rusts of several cultivars is based on the slow rusting genes having additive effects (Singh *et al*., 2005). The most efficient and economical management of wheat rusts is the generation of rust resistant varieties and their on-farm cultivation (Chaudhary *et al*., 1998; Hussain *et al*., 1999; Kalappanavar *et al*., 2008). In the present era of scientific advancement, the wheat research is focused to

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achieve durable rust resistance through incorporation of multiple minor genes or adult plant resistance genes (Broers, 1989; Singh and Rajaram, 1991; Singh *et al*., 2000; Singh *et al*., 2005; Rehman *et al*., 2013). The worst yellow rust epidemics in 2005 and 2012 have wiped out major commercial wheat varieties of Pakistan (Khan *et al*., 2005; Hussain *et al*., 2015). Yellow rust (*Puccinia striiformis* f. sp. *tritici*) can reduce wheat yields by as much as 84% (Murray *et al*., 1995). Rusted plots yielded 4% less crops as compared to fungicide-protected plots for cultivars with hypersensitive resistance (Singh and Rajaram, 1991). Yield and kernel weight average of rusted plots are 8% less for cultivars with partial resistance, but depending on cultivar, varied from 2 to 20% less. Yield losses in wheat caused by leaf rust in cultivar trials were estimated at five locations in Mississippi from 1986 to 1989 (Khan *et al*., 1997). At present country is facing critical shortage of appropriate wheat varieties having both features of high yield and rust resistance to leaf, yellow and stem rust (Hussain *et al*., 2006). Now a days, pathologists and breeders have sought resistance mechanism based on minor genes which is called durable rust resistance or adult plant resistance (Broers, 1989; Singh and Rajaram, 1991; Singh *et al*., 2000). This type of rust resistance mechanism is more effective for many races rather than a single one and is long lasting (Hussain *et al*., 1998; Hussain *et al*., 1999; Bariana *et al*., 2001). A high level of resistance (approaching immunity) to yellow rust could be achieved by accumulating 4 to 5 minor genes in a variety (Singh *et al*., 2005). However, moderate level of resistance can be achieved by accumulating 2–3 minor genes in a line (Singh *et al*., 2005). In spite of the absence of any effective major gene, the partial resistance of varieties indicated the presence of minor genes (Hussain *et al*., 2006). Parents having partial resistance are crossed to pyramid genes for rust resistance and yield. This resulted many wheat lines that were better in yield and disease resistance as compared to their parent (Hussain *et al*., 2007). This, in addition, results in diversification of wheat genotypes in terms of their resistance background, necessary to avoid rapid evolution of the rust pathogen to acquire new virulence. Genetic resistance of leaf, yellow and stem rust resistant varieties is being considered the only remedy to prevent the crop from diseases as the long-term strategy. Hence, the objective of this study was to develop germplasm for wheat cultivars having minor gene based resistance against leaf yellow and stem rust along with characters for high yield.

Materials and Methods

The research work presented here was carried out at Wheat Research Institute (WRI) and Agriculture Biotechnology Research Institute (ABRI), Faisalabad during the year 2011–12, for the selection and molecular characterization of wheat breeding material. It involved screening of wheat germplasm against leaf and yellow rust under natural and high stress inoculation conditions for selecting the lines that may show minor gene based resistance followed by transfer of this resistance to the susceptible but high yielding varieties through conventional hybridization utilizing genotypic markers as used elsewhere for the identification of genes for pyramiding rust resistance genes in bread wheat genotypes and commercial cultivars (Khan, 1987; Dakouri *et al*., 2013). Newly available DNA markers X-barc 352, XWMC-44, Xgwm-533 for and *Lr34/Yr18*, *Lr46/Yr29* and *Sr2/Yr30* respectively were used to assist the selection of wheat germplasm with desirable genes (Sharp *et al*., 2001; Suenaga *et al*., 2003; William *et al*., 2003). Researchers have confirmed effectiveness of *Lr34/Yr18, Lr46/Yr*29 and *Sr2/Yr30* genes against rusts (McIntosh, 1992; Singh, 1992).

Selection of Breeding Material

The material used for the crossing was selected on the basis of higher grain yield among wheat lines from the gene pool of WRI. 6–10 year rust history of the lines was also considered for final selection of the parents. Two hundred and twenty lines were selected from 750 lines for molecular characterization. The selection was based on low terminal rust reactions up to 20–30 MRMS in slow rusting response.

Data Recording of Leaf and Yellow Rust

Rusts data were recorded at every 10 days interval. The rust severity and field response were recorded according to modified Cobb's scale described by Peterson *et al*. (1948). Severity was recorded on the basis of percentage and field responses. Severity ratings were based upon visual observations recorded at 10 days intervals as trace to 5, 10, 20, 40, 60 and 100 percent infection and field response as immune, resistant, moderately resistant, intermediate, moderately susceptible and susceptible by the scale given in Table 1. Three observations regarding rust severity were recorded before the physical maturity of the crop. Three hundred and forty five desirable lines were selected on the basis of rust reaction. The other parameters like Plant height, Grain yield assessment ha^{-1} and 1000 grain weight and protein percentage were also recorded. The disease severity data were used to calculate the area under the disease progress curve (AUDPC).

$$AUDPC = \Sigma i\ [(xi + x\ i+1)/2]ti,$$

Where xi is the severity value on date i, ti the time interval in days between two consecutive evaluations dates i and i + 1 (Chen and Line 1995a). Also the area under disease progress curve (AUDPC) was worked out by using software developed by CIMMYT (Jeger *et al*., 2001). The acceptable range of AUDPC for leaf rust is 300 and for yellow rust is 200 as yellow rust appears earlier in the season and can cause more losses than leaf rust. All those lines falling above these ranges were discarded out of the evaluation of resistance genes through marker application.

Molecular Characterization and Yield Testing of 220 Selected Lines Through Markers Applications

For molecular characterization of 220 selected advanced lines for rust resistance was done by using three reliable molecular markers i.e. Xgwm-533, X-barc-352 and XWMC-44 (Sharp *et al*., 2001; Suenaga *et al*., 2003; William *et al*., 2003). This research work was conducted at the Agricultural Biotechnology Research Institute, AARI, Faisalabad.

Sample Collection

For DNA extraction, fresh leaves from 220 genotypes of wheat were collected from the Wheat Research Institute, Ayub Agriculture Research Institute, Faisalabad Pakistan. The fresh leaves were collected for sufficient DNA yield. After tagging of samples, the leaves were washed with distilled water and transferred in liquid nitrogen chamber available in Agricultural Biotechnology Research Institute (ABRI) and were kept on -80°C for avoiding degradation of leaf samples.

DNA Extraction

Grinding was given to plant samples in liquid nitrogen to break cell walls, CTAB, was added to release DNA from the cell and nuclear membranes and chloroform was added to make DNA sample protein free. For further purification of the DNA sample, other reagents like RNase, NaCl are also added and centrifuged for repeated times to finally obtain DNA in the form of pellets. Before starting DNA isolation, the water bath was turned on and the temperature was set at 65°C and preheated 2X CTAB with mercaptoethanol in the water bath.

Molecular Markers Analysis

For the SSR analysis concentration of genomic DNA, 10X PCR Buffer with $MgCl_2$, $(NH_4)_2SO_4$, dNTPs (dATP, dCTP, dGTP, dTTP), 10mer *SSR* primer and Taq DNA polymers were optimized. The *SSR* primers obtained from Gene Link Company (USA) were used for the amplification of the genomic DNA. Taq Polymerase, Buffer, $MgCl_2$ and dNTPs were purchased from Fermentas, USA. Polymerase chain reactions (PCRs) were carried out in eppendorf thermal cycler. The following concentrations of PCR reagents were used in the experiment, (Table 2). Amplification reaction profile for PCR analysis are given in Table 3–5.

Protocol for Agarose Gel

An amount of 10.5 g agarose was weighed in the electronic balance and dissolved in 350 mL 1X TBE buffer. It was heated for about 3 min and left to cool under running tap water. Then 15 µL of ethidium bromide was added and mixed gently. The gel was poured slowly into the tank. Any bubbles were pushed away to the side using a disposable tip. The combs were inserted in it. The sizes of combs were selected according to the requirement, i.e. 23 wells, after which the gel was left to set. Having the gel solidified, the 1XTBE buffer was added to the gel tank to submerge the gel to 2–5 mm depth. The first well was loaded with marker/ladders with the amount of 5 µL. An appropriate amount of about 10 µL + 3 µL dye of each sample were loaded in other wells (Table 6). The gel tank was closed and the gel was run by supplying some suitable amount of current i.e. 90 volts. After electrophoresis, the amplified products were viewed under ultraviolet transilluminator and photographed using Gene Snap software on Synvgene Gel Documentation System (GDS) USA.

Results

Molecular Characterization and Yield Testing of 220 Selected Lines Through Markers Applications

Among 220 entries under yield, 99 entries were found to be high yielding ranging from 3973–4786 kg/ha, 71 entries showed height ranging from 100 to 120 cm, 34 entries showed higher 1000 grain weight ranging from 42 to 47 grams and 50 entries had higher protein %, which ranged from 12–14 percentage and their rust response on the basis of AUDPC as compared to check varieties. Among 220 outstanding lines of wheat against leaf rust during the studies 137 lines exhibited AULRPC values less than 300 and rate of rust development was very slow. Only 83 entries had high levels of AULRPC rating from 325 to 950 and may be discarded in any variety development program. Similarly, among 220 outstanding lines of wheat against Yellow rust during the studies183 genotypes exhibited AUYRPC values less than 200 and rates of rust development was also very slow. Only 37 entries had high levels of AUYRPC rating from 225 to 700. These genotypes would be a good source for future wheat hybridization program in the country to achieve higher yield and high resistance as reported by many research workers (Ezzahiri and Roelfs, 1989; Singh, 1992; McIntosh, 1992; Maqsood *et al*., 2000). Among tested entries, 67 entries showed *Lr34/Yr18*, 23 entries showed *Lr46/Yr29* and 62 entries showed *Sr2/Yr30* the presence. The results for identification of durable rust resistant genes were inferred on the basis of genotypic expressions (Table 8). While the rust resistance genetics were verified through amplification of molecular markers. The markers, Xgwm-533 for *Sr2/Yr30*, X-Barc352 for *Lr34/Yr18* and Xwmc-44 for *Lr46/Yr29* were applied to the selected 220 genotypes and the results indicated that.

Nineteen wheat genotypes showed the presence of leaf rust resistance genes *Lr34/Yr18* with the fragment size of 250 bp and only 22 advance lines were amplified by PCR in which nineteen elite lines were resistant and three V-11192,

Table 1: Disease rating scale used to record rust severity and level of resistance/susceptibility of wheat varieties

	Field Response	Symptoms
0	Immune	No visible infection
R	Resistant	Visible chlorosis or necrosis, no uredia are present
MR	Moderately Resistant	Small uredia are present and surrounded by either chlorotic or necrotic areas
M	Intermediate (Mixed)	Variable sized uredia are present some with chlorosis, necrosis or both
MS	Moderately susceptible	Medium sized uredia are present and possibly surrounded by some chlorotic areas
S	Susceptible	Large uredia are present, generally with little or no chlorosis or necrosis
(Peterson *et al*., 1948)		

Table2: Concentrations and volume of reagents used in *SSR* markers analysis

Reagents	Concentration	Volume
Double distilled water (d_3H_2O)	-	5.6 to 9.5µL
Buffer $MgCl2+(NH_4)_2SO_4$	10X	1.5 to 2.0µL
dNTPs	50 mM	0.75 to 1.50µL
Taq Polymerase	0.2 mM	3.0µL
Primer (forward+reverse)	5 unit/µl	0.2 to 0.6µL
Template DNA	15ng/µl	1.5+1.5 µL
Total volume 20 µL	30ng/µl	2.0 to 5.0µL

Table 3: Amplification reaction profile for PCR analysis (*LR34/YR18* X-BARC 352)

Steps	Temperature	Time
1st	94°C	5 minutes (denaturation)
2nd	94°C	30 seconds (denaturation)
3rd	60°C	30 seconds-1 minute (annealing)
4th	72°C	30 seconds (extension)
5th	Go to 2nd	Repeat 38 cycles
6th	72°C	5 minutes (Final extension)
7th	20°C	Hold until the tubes are removed

Table 4: Amplification reaction profile for PCR Analysis (*LR46/YR29* Xwmc-44)

Steps	Temperature	Time
1st	94°C	5 minutes (denaturation)
2nd	94°C	1 minutes (denaturation)
3rd	55°C	1 minute (annealing)
4th	72°C	2 minute (extension)
5th	Go to 2nd	Repeat 45 cycles
6th	72°C	10 minutes (final extension)
7th	20°C	Hold until the tubes are removed

V-11202 and V-11210 were found susceptible (Fig. 1).

Lr34/Yr18 X-barc 352: Molecular marker X-barc 352 was found to be linked with *Lr34/Yr18*. All wheat genotypes showed the presence of leaf rust resistance genes *Lr34/Yr18* with the fragment size of 250 bp and PCR amplified only 21 advance lines in which all the elite lines were resistant except one V-11220 (Fig. 2).

*Lr46/Yr*29 XWMC-44: Molecular marker XWMC-44 showed linkage to leaf rust resistance gene *Lr46/Yr29*. Five elite lines were resistant and fifteen i.e. V-11191, V-11194, V-11197, V-11198 V-11199, V-11200 V-11201, V-11202, V-11203, V-11204, V-11205, V-11206, V-11207, V-11208 and V-11209, were found susceptible against *Lr46/Yr29* gene with the fragment size of 242 bp and amplification of only 20 advance lines by PCR has been illustrated (Fig. 3).

*Lr46/Yr*29 XWMC-44: Molecular marker XWMC-44 showed linkage to leaf rust resistance gene *Lr46/Yr29*. Three elite lines were resistant and seventeen i.e. V-11210, V-11212, V-11213, V-11214 V-11215, V-11216 V-11217, V-11218, V-11219, V-11220, V-11221, V-11223, V-11224, V-11225 and V-11226 were found susceptible (Fig. 4).

Sr2/Yr30 XGWM-533: Molecular marker (Xgwm-533) was found to be linked to stem rust resistance gene *Sr2/Yr*30. All wheat genotypes showed the presence of stem rust resistance genes *Sr2/Yr30* with the fragment size of 120 bp. Only 22 lines were amplified by PCR (Fig. 5) in which 17 lines were resistant and five i.e. V-11191, V-11202, V-11204, V-1125 and V-11207 were found susceptible.

Sr2/Yr30 XGWM-533**:** Molecular marker (Xgwm-533) was found to be linked to stem rust resistance gene *Sr2/Yr30.* All wheat genotypes showed presence of Stem rust resistance genes *Sr2/Yr30* with the fragments size of 120 bp and the amplification of only 22 advance lines has been illustrated (Fig. 6) in 21 lines were resistant and one V-11217 was found susceptible.

It was concluded that out of 220 genotypes, only nine genotypes V-11211, V-11227, V-11288, V-11296, 11304, V-11308, V-11319, V-11353 and V-11396 showed the combination of three designated slow rusting/durable genes, along with high yield, 1000 grain weight, protein % and plant height ranging from (100 to 120 cm). This is very important combination, as it provides protection against three types of rusts (*LR*, *YR* and *SR*), while 15 genotypes including V-11203, V11212, V-11218, V-11223, V-11245, V-11248, V-11250, V-11262, V-11267, V-11289, V-11321, V-11232, V-11338, V-11365 and V-11359 showed the combination of *Sr2/Yr*30 and *Lr34/Yr18*. Similarly, the combination of *Sr2/Yr30* and *Lr46/Yr29* was found in 2 genotypes including V-11190, V-11193 and the combination of *Lr46/Yr29* and *Lr34/Yr18* was found in 6 genotypes including V-11276, V-11247, V-11313, V-11345, V-11376 and V-11380 (Table 9).

Discussion

Among the tested genotypes, V-11211, V-11212, V-11218, V-11227, V-11262, V-11288, V-11296, V-11304, V-11308, V-11319, V-11338, V-11353, V-11365 and V-11396 showed the combination of three designated slow rusting/durable genes; *Sr2/Yr30, Lr46/Yr29* and *Lr34/Yr18*,

Table 5: Amplification reaction profile for PCR analysis (*SR2/YR30* Xgwm-533)

Steps	Temperature	Time
1st	94°C	5 minutes (denaturation)
2nd	94°C	1 minutes (denaturation)
3rd	60°C	1 minute (annealing)
4th	72°C	2 minutes (extension)
5th	Go to 2nd	Repeat 45 cycles
6th	72°C	10 minutes (Final extension)
7th	20°C	Hold until the tubes are removed

After PCR amplification, the concentration of amplified genotype was determined on 3.0% (w/v) high resolution agarose gel (Table 6) prepared in TBE stained with Ethidium bromide

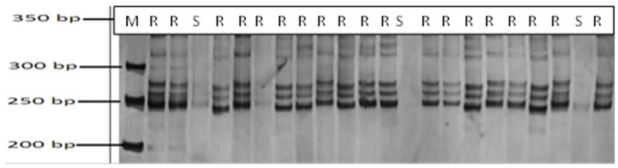

Fig. 1: PCR amplification products resolved in polyacrylamide gels using for 220 lines from elite material which produced resistant and susceptible bands by the size marker (X-barc 352)

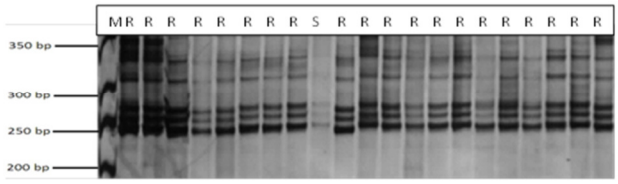

Fig. 2: PCR amplification in polyacrylamide gels when using X-barc 352 on 220 lines from elite material which produced resistant and susceptible fragments by the size marker (X-barc 352)

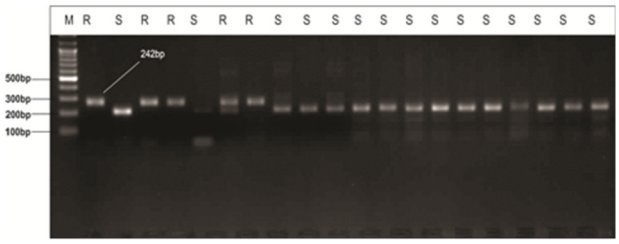

Fig. 3: PCR amplification products resolved in acrylamide gels using for 220 elite lines from elite material which produced resistant and susceptible bands by the size marker (XWMC-44)

along with high yield, 1000 grain weight, protein % and plant height (ranging from 100 to 120 cm) carried resistance near immunity under the severe leaf and yellow rust severity conditions. According to Singh *et al*. (2005) a high level of resistance (approaching immunity) to yellow rust could be achieved by accumulating 4 to 5 minor genes in a variety. However, moderate level of resistance can be achieved by accumulating 2–3 minor genes in a line (Singh *et al*., 2005). CIMMYT and AARI planned a strategy of pyramiding APR genes alone or in combination with major genes to combat the recently emerged races of stem and yellow rust (Rehman *et al*., 2013). These lines may be a valuable source of rust resistance with amber grain color. The resistance in the derived lines seems to be race non-specific and durable nature. The major genes possessed by the parents were susceptible as the individual line V-87094 had high terminal rust rating up to 80% in the rust screening nurseries and variety Era exhibited 10–20% rust rating. Combinations form these parents against the prevalent leaf and yellow rust races showed fairly very low rust intensity in the country (Hussain *et al*., 2006). The lines which possessed major genes individually are susceptible for most rust virulences in Pakistan. Although the rust development was slow in case of *Lr34*, but alone this gene did not give desired protection and terminal rust rating was more than 60%. The better resistance in the derivatives from these crosses was most probably through the pyramiding of additional minor genes in their ancestors (Hussain *et al*., 2010; Hussain *et al*., 2011). Most of the lines were resistant to moderately resistant to leaf and yellow rust under high leaf and yellow rust inoculums pressure, developed artificially at the WRI, Faisalabad. The spreader rows of susceptible Morocco were full of rust rating 80–100 SN and there was no chance of escape. The year 2012 was the worst epidemic year for yellow rust, wiped out most of the wheat cultivars of the country including Seher-2006, MH-97 and Bakhar-2002. Only Faisalabad-2008, Lasani-2008, AARI-11, Millat-11 and Pb-11 were found relatively resistant (Hussain *et al*., 2015). Therefore, a mechanism based on the additive effects of partial resistance minor genes and probably different from all the existing wheat varieties of Pakistan would be useful in breeding against rust resistance. This kind of resistance is desirable, as it is long lasting, more durable against changing rust virulence patterns. This is evidently supported by the consistent resistance response of the varieties Frontana and Era in Pakistan for the last twenty years (Hussain *et al*., 1999) and hence high economic returns may be achieved from such kind of resistance. Such findings and ideas have been emphasized, entrusted and floated by many researchers (Chaudhary *et al*., 1998; Singh *et al*., 1998, 2000; Hussain *et al*., 1999; Navabi *et al*., 2000).

Some new forms of rust virulence have been generated as a result of mutations in the nature. New rust virulence is appearing with the introduction of new wheat varieties and many wheat varieties have been banned for commercial cultivation only due to rust susceptibility against new rust virulence (Khan *et al*., 2002). Incorporation of more than one gene to cultivars for durable leaf rust resistance has remained the focus of the breeders to cope with the dynamic nature of the pathogen (Roelfs, 1988). To address this issue, gene postulation as well as molecular marker approach is being utilized for enhancing rust resistance mainly through identification of durable rust resistance gene and

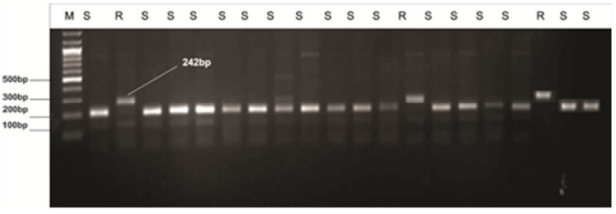

Fig. 4: PCR amplification products resolved in acrylamide gels using for 220 elitlines from elite material which produced resistant and susceptible bands by the size marker (XWMC-44)

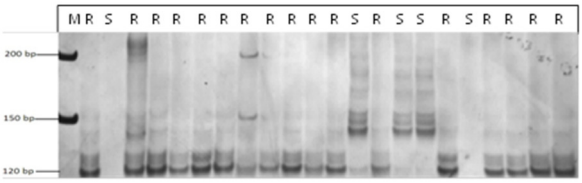

Fig. 5: PCR amplification products resolved in polyacrylamide gels when using for 220 lines of elite material which produced resistant and susceptible fragments by the size marker (Xgwm-533)

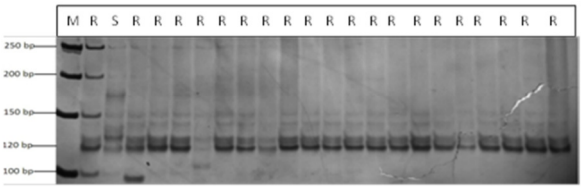

Fig. 6: PCR amplification products resolved in polyacrylamide gels when using for 220 lines of elite material which produced resistant and susceptible fragments by the size marker (Xgwm-533)

pyramiding different seedling and adult plant resistance genes. Considering this, wheat genotypes postulated to carry *Lr34* gene were screened with microsatellites and STS marker (*csLV*34) which were found to be a useful as molecular marker and *Lr34* gene were reported to be linked closely (Lagudah *et al*., 2006). In Pakistan, scientists postulated host material of Pakistani wheat for *Lr* family of leaf rust resistance genes including *Lr34* and confirmed its presence in local genetic stocks (Rattu, 2010). Also it has been examined in 39 isogenic wheat lines and 12 commercial cultivars from Pakistan at different locations and observed virulence of *Lr34* (Fayyaz, 2008). In present investigation, the molecular characterization of 220 genotypes through the application of molecular markers i.e. Xgwm-533 for *Sr2/Yr30*, X-Barc352 for *Lr34/Yr18* and Xwmc-44 for *Lr46/Yr29* revealed that 67entries showed presence of *Lr34*/*Yr18*, 23 entries showed *Lr46*/*Yr*29 while 62 entries showed *Sr2*/*Yr30* linkage. Cultivars possessing slow rusting illustrated lower AUDPC at adult plant stage have race-nonspecific resistance as also described by (Sandoval-Islas *et al*., 1998 and Singh *et al*., 2005). Because the durable resistance, like slow rusting and high-temperature adult plant resistance is polygenic (at least 2-3 controlling genes) as described elsewhere (Dehghani and Moghaddam, 2004), therefore, it remains successful for longer time, even if the pathogen under goes mutation. Hence, as per our findings, lines showing low frequency of disease severity with lower AUDPC values could be considered as slow rusting lines carrying durable rust resistance against *Lr34*, *Lr46* and *Sr2* virulences, which can be utilized in breeding programs. For its relative ease, specificity and efficiency, many authors have employed PCR-based DNA markers to verify presence of leaf rust resistance in wheat (Cherukuri *et al*., 2003; Prabhu *et al*., 2004; Obert *et al*., 2005; Lagudah *et al*., 2006; Dakouri *et al*., 2013; Mustafa *et al*., 2013). Also it requires no laborious means or to wait for particular plant stage to observe time bound gene expression controlling trait of interest i.e. adult plant resistance. Therefore, for further justification of leaf rust resistance estimation, *Lr34* linked sequence tagged sites (STS) molecular marker (*csLV*34) were used to mark the presence or absence of the gene in landraces and cultivars.

A relatively smaller size amplicon of less than 100 bp was amplified in remaining 9 genotypes which is considered as marker for susceptible allele as that revealed that a known fragment of 79 bp (insertion in intron) is found to be linked to leaf rust susceptibility in bread wheat gene (Lagudah *et al*., 2006). However, when molecular data was compared with field study, we observed two sets of observations; first type included those genotypes whose molecular data for *Lr34*, *Lr46* and *Sr2* presence corresponded well with the field data i.e. performance of genotypes in the field for avirulence pattern. The genotypes that showed the presence of 250 bp band for *Lr34* exhibited moderately resistant to moderately susceptible (Shah *et al*., 2010; Priyumvada *et al*., 2009). Similarly the genotypes which failed to show the presence of 250 bp band for *Lr34* gene and were susceptible to leaf rust in the field. Hence, the absence of this gene as revealed through marker data corresponded well with the expression data in the field (Lagudha *et al*., 2006).

In the second category of observation both the data sets did not match with each other i.e. some of the genotypes showed the presence of *Lr34* in the molecular analysis but in the field they remained susceptible to leaf rust. This contrast in the experimental and field results may be due to random mutations, suppression or deletion or evolution of new pathotype could also be the possible reason of inability of the wheat lines to cope with the avirulences (Awan *et al*., 2007; Dakouri *et al*, 2013). Some of the genotypes did not reveal the presence of *Lr34* locus when screened through molecular marker although in the field those lines exhibited moderate resistance to leaf rust. These type of discrepancies have been reported in the recent past, presence of *Lr34*was indicated in popular wheat cultivars "Cappelle Desprez" on the basis of observed genetic association of leaf and stripe rust

Table 6: Preparation of 0.8% agarose gel

1	Weigh 2.0 g of high resolution gel.
2	Measure 100 mL of 1.0Xtbe buffers in a conical flask, and add weighted agarose in flask.
3	Weigh this flask by keeping it on electric balance.
4	Keep the flask in oven for 3 minutes.
5	Reweigh it after boiling and adjust the original weight by adding distilled Water in it.
6	Add 3ul ethidium bromide (fluorescent dye) in this solution.
7	Cool the flak with running tap water till the room temperature (37ºC) is attained, pour it into a gel tray keeping in the casting tray (remember to balance the casting tray before pouring gel into it.
8	Insert comb in the gel of required size and teeth.
9	Leave it for some time to allow polymerization of gel.
10	Fill the electrophoresis tank with 1.0 x TBE buffer before keeping the polymerized gel into it.
11	After keeping gel into buffer, pull out the comb/s gently.

Table 7: Selection of single headlines from F_6 generation crosses

Name of the cross	Tested entries	Selected entries
FRT/SA42//PRL/SA42/4/Pfau/SERI.1B//Ammad/3/Waxwing	20	12
FRT/SA42//PRL/SA42/3/Wblli2*/Brambling	22	13
FRT/SA42//PRL/SA42/3/Kiritati	24	16
Wattan/2*ERA/2/Pak-81/2*Wattan/3/Shafaq-06/4/Brambling	37	24
Wattan/2*ERA/2/Pak-81/2* Wattan /3/Shafaq-06/3/Wblli/ Brambling	21	12
Wattan /Fsd-08//Kiritati	29	12
Pak-81 2*/Wattan//2*Shafaq-06/3/Kiritati	35	16
Pak-81 2*/Wattan//2*Shafaq-06/3/Juchi F2000	21	7
Pak-81 2*/Wattan//2*Shafaq-06/3/Dollarbird	24	9
Pak-81 2*/Wattan//2*Shafaq-06/3/Kambi/2*Khawaki	23	6
Luan/Kohistan/Pak81/3/Kiritati	42	24
Wattan/2*ERA/2/Pak-81/2*Wattan/3/Shafaq-06/4/Kiritati	26	14
Wattan/2*ERA/2/Pak-81/2*Wattan/3/Shafaq-06/4/Kingbird	22	6
SH88/90A204//MH-97/3//PRL/2*Pastor	20	9
Wattan/2*ERA//Lasani-08	22	12
Shafaq-06/ Luan// MH-97	34	19
Uqab-2000/ Wattan/*Lr28*//Yecora-70	37	22
SH88/ Pak-81// MH97/3/ Shafaq-06	27	16
Wattan/ 2*ERA// V04178	35	17
Wattan/ 2*ERA// V03007	42	14
Wattan/ 2*ERA// V04179	25	6
Wattan/ 2*ERA// Wattan/ *Lr28*//Yecora-70	28	6
Lasani-08/Seher-06	38	10
Lasani-08/Iqbal-2000	27	14
Lr19/V02192// Shafaq-06	56	27
Total	750	343

Table 8: Genotypes showing slow rusting linkage

Genotypes		Geenotypic Markers			Units of AUDPC ranging	
V-Codes	Total	*Lr34/Yr18* (X-barc 352)	*Lr46/Yr29* (XWMC-44)	*Sr2/Yr30* (Xgwm-533)	*Lr*	*Yr*
V-11195, V-11196, V11211, V11222, V-11227, V-11230, V-11231, V-11288, V-11296, 11304, V-11308, V-11319 V-11353 and V-11396	14	+	+	+	0-200	0-175
V-11194, V11198, V11200, V-11203, V11207, V-11208, V11209, V11212, V11215, V11216, V-11218, V-11219, V-11221, V-11223, V11224, V11225, V-11226, V11228, V-11229, V-11232, V-11244, V-11245, V-11248, V-11250, V-11262, V-11263, V-11267, V-11270, V-11280, V11282, V-11289, V-11307, V-11321, V-11328, V-11232, V-11329, V-11333, V-11337, V-11338, V-11340, V-11356, V-11359, V-11367, V-11375, V-11390 and V-11392	46	+	-	+	0-325	0-225
V-11190 and V-11193	2	-	+	+	25-100	25-200
V-11276, V-11247, V-11290, V-11313, V-11345, V-11376 and V-11380	7	+	+	-	0-325	0-275

resistance (McIntosh, 1992) but in a stark contrast was not observed using resistance gene specific marker (Lagudah *et al*., 2009). Besides, presence of other rust resistant gene(s) could also be the possible reason of plants resistance against disease as is the case of new leaf rust resistant gene (*Lr67*) which is almost similar in many characteristics to *Lr34* (Spielmeyer *et al*., 2013). Also, PCR failure to amplify the particular band during amplification could be another probability of inconsistency between field and molecular marker data (Ali *et al*., 2007).

Determining the presence of *Lr34*, *Lr46* and *Sr2* in current cultivars can be helpful to predict the field resistance

Table 9: Elite lines with combination of three designated slow ruster, durable resistant genes, high in yield, grain weight, proteins percentage and height

Selection	V-Code	*Lr34/Yr18*	*Lr46/ Yr29*	*Sr2/Yr30*	kg/ha	1000-grain wt	height in cm	Protein %
119	11308	+	+	+	4786	44	109	10
23	11212	+	-	+	4780	45	119	10
73	11262	+	-	+	4749	34	113	10
130	11319	+	+	+	4737	47	115	11
115	11304	+	+	+	4663	42	114	10
78	11267	+	-	+	4577	37	117	10
31	11220	-	-	+	4035	39	113	11
38	11227	+	+	+	4558	34	118	13
34	11223	+	-	+	4558	37	119	10
100	11289	+	-	+	4552	36	124	10
176	11365	+	-	+	4550	41	112	10
61	11250	+	-	+	4539	38	110	11
149	11338	+	-	+	4517	35	119	12
107	11296	+	+	+	4515	33	107	10
56	11245	+	-	+	4502	40	120	12
59	11248	+	-	+	4490	33	115	12
207	11396	+	+	+	4488	41	118	11
29	11218	+	-	+	4484	37	120	12
164	11353	+	+	+	4467	34	110	11
58	11247	+	+	-	4465	36	114	10
99	11288	+	+	+	4453	37	124	12
132	11321	+	-	+	4416	40	137	12
211	11400	-	-	-	4414	37	129	12
131	11320	+	-	-	4391	37	122	12
32	11221	+	-	+	4360	44	113	10
43	11232	+	-	+	4360	41	136	12
87	11276	+	+	-	4317	46	118	11
140	11329	+	-	+	4270	35	120	13
170	11359	+	-	+	4270	38	111	13
14	11203	+	-	+	4262	38	107	12
91	11280	+	-	+	4256	44	113	12
136	11325	+	-	-	4245	43	119	11
201	11390	+	-	+	4241	41	109	10
2	11191	+	-	+	4237	33	133	14
124	11313	+	+	-	4231	47	129	11
37	11226	+	-	+	4225	44	120	12
70	11259	-	-	+	4218	33	103	9
218	11407	-	+	-	4216	36	101	11
28	11217	+	-	+	4212	46	106	9
166	11355	-	-	-	4208	43	112	9
90	11279	-	+	-	4194	45	120	12
93	11282	+	-	+	4194	45	120	12
4	11193	-	+	+	4175	31	110	12
220	11409	-	-	-	4167	30	108	10
167	11356	+	-	+	4159	41	122	12
72	11261	-	-	+	4157	34	109	9
88	11277	-	-	-	4157	36	110	12
186	11375	+	-	+	4118	42	104	12
156	11345	+	+	-	4085	40	110	11
163	11352	-	-	-	4085	44	100	10
135	11324	+	-	-	4072	36	117	12
96	11285	-	-	-	4070	39	121	13
184	11373	-	-	-	4068	42	112	13
122	11311	-	-	-	4058	42	121	11
92	11281	-	-	+	4046	44	137	11
191	11380	+	+	-	4031	38	114	10
1	11190	-	+	+	4027	42	113	12
161	11350	-	+	-	4023	42	113	11
169	11358	-		-	4023	36	102	10
187	11376	+	+	-	4019	38	102	11
152	11341	-	+	-	3986	43	111	10
39	11228	+	-	+	4311	36	116	10
139	11328	+	-	+	4295	35	19	10
140	11329	+	-	+	4270	34	120	13
22	11211	+	+	+	4484	37	120	11

+ Sign shows the presence of rust resistance genes in wheat genotypes while
- Sign shows absence of rust resistance genes in wheat genotypes

and durability of these cultivars and to aid decisions in selecting parents for future breeding and development of new and improved cultivars with improved leaf rust resistance. Therefore, the strategy of incorporating partially resistant minor gene in wheat genotypes through hybridization is the best way to achieve long lasting resistance in the wheat cultivars under the changing pattern of rust races/virulence in the country.

Conclusion

Nine lines i.e., V-11211, V-11227, V-11288, V-11296, V-11304, V-11308, V-11319, V-11353 and V-11396 showed the combination of three designated slow ruster/durable resistant genes (*Sr2/Yr30, Lr46/Yr29 and Lr34/Yr18*). Fifteen lines including V-11203, V11212, V-11218, V-11223, V-11245, V-11248, V-11250, V-11262, V-11267, V-11289, V-11321, V-11232, V-11338, V-11365 and V-11359 showed the combination of *Lr34/Yr18* and *Sr2/Yr30*. Two lines including V-11190, as well as V-11193 showed the combination of *Lr46/Yr29* and *Sr2/Yr30*. Six genotypes including V-11276, V-11247, V-11313, V-11345, and V-11376 and V-11380 showed combination of *Lr46/Yr29* and *Lr34/Yr18*. These outstanding lines having high level of partial resistance along with lower AUDPC may be used in breeding program to transfer its partial/durable resistance character to the adapted wheat cultivars/varieties of Pakistan (Inqilab-91, MH-97, Wattan, Pb-96, Seher-2006 and Shafaq-2006 etc).

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