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DEVELOPMENT OF PUTATIVE MOLECULAR MARKERS TO TRACE DURABLE RUST RESISTANCE GENES IN WHEAT BREEDING STOCKS

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Abstract

Wheat plays a central role in Pakistan's food economy in terms of production and consumption. Wheat shares 3.1% of national GDP. Rusts are among the devastating diseases of wheat. A large number of rust resistance genes are known in wheat for stripe (yellow), leaf (brown) and stem rust. Selection for durable resistance in rust breeding programs is typically carried out in field screening nurseries or under controlled conditions only for advanced breeding lines. Marker Assisted Selection (MAS) can be useful under such conditions. Availability of small number of DNA markers for identification of genes involved in rust resistance makes MAS application difficult. Identification and designing of new PCR primers may help to select rust resistant genotypes from wheat breeding stocks in early segregating generations. This study is designed with aim to identify and design PCR primers for resistance genes in wheat breeding stocks. Data and sequences were retrieved through extensive literature and databases search. Data mining was done on public domain available databases i.e., NCBI nucleotide, Grain Genes and Plant GDB. A total of 12 sequences for leaf rust resistance, 13 for stripe rust resistance and 2 for stem rust resistance were retrieved. Homology studies, motif finding and their phylogenetic analysis was done to infer their possible functions and to develop PCR primers to mark the rust resistance genes in wheat germplasm. These markers were validated for detection of rust resistance gene among 16 local varieties, which proved valuable for future wheat breeding.

Introduction

In Pakistan, wheat is a main Rabi crop and staple food crop. It accounts for nearly 38% of the total cropped area and 30% of the value added by major crops (Coleman & Faruqee, 1996; Alam, 2001). Pakistan ranks 6th in wheat production, 8th in area, however, 59th in terms of yield (Ashfaq *et al*., 2011). Wheat also plays a central role in the economy of Pakistan. Share of wheat in the GDP of Pakistan is 3.0% (Accessed from http://par.com.pk/2012/04/11/wheat-field-visit-6-april-2012/, on 20th April.)

Wheat crop endures from several biotic and abiotic stresses including diseases, pests, drought and heat throughout its life cycle, which causes substantial yield losses. Wheat rusts (leaf, stem and stripe rust) caused by *Puccinia* are among the major production constraints worldwide (Khan *et al.*, 2012). In Pakistan, 1972 and 1973 leaf rust epidemics' severity was 20-30% and 40-50% respectively, while the epidemics of 1976 and 1978 increased to 50-80% severity level with 30% yield losses (Khan, 1997). Yellow rust epidemics during 1995 and 2005 caused significant yield losses (Khan *et al.,* 2005). Bhatti & Bhutta (2002) recorded many seed borne pathogens from infected wheat seeds collected from the various agro-ecological regions of Pakistan. Akhtar *et al.,* (2010) reported the grain yield losses in wheat genotypes tested in Bahawalpur due to the Russian wheat aphid. Therefore, wheat's survival depends on the ability to sense these pathogens and generating a strong defense response. Wheat relies on innate immunity, such as the ability of each cell to identify the pathogen, and originate signals from the infection site as a result of pathogen invasion (Rivas, 2012; Rasheed *et al.,* 2012).

In wheat, disease resistance can be classified in two broad categories; complete or race specific resistance conferred by single or major gene and partial or race non-specific resistance conferred by many genes (Nagori, 2009). Race specific resistance leads to the hypersensitive response (HR) and inhibition of pathogen growth. HR includes the rapid oxidative burst, ion fluxes, phytoalexins accumulation and induction of various pathogenesis related proteins (Staskawicz *et al.,* 1995). Non-specific resistance is conferred by many minor genes having additive effect. It is also termed as durable resistance or adult plant resistance. Durable resistance is an effective type of resistance against the newly emerging rust races in the wheat (Qamar *et al.,* 2007; Hussain *et al.,* 2011a). Singh *et al.,* (2004) suggested that durable rust resistance is controlled by more than single gene, by pyramiding 4 or 5 minor genes. It is more likely to operate at the adult plant stage rather than the seedling stage, and is thought to confer non-hypersensitive response to infection.

There are various durable resistance genes identified in wheat i.e., Lr34/YR18 (Lagudah, 2005), Lr46/Yr29 (William *et al.,* 2003), Sr2 (Chalmers *et al.,* 2004) and lr67/Yr46 (Bariana *et al.,* 2011) Among these Lr34/Yr18 is most valuable region for disease resistance breeding. Lr34 encodes a putative ABC transporter. Lr34 is located in the chromosome 7D of wheat (Singh, 1992). This dual APR gene also co-segregates with other traits i.e., leaf tip necrosis, powdery mildew resistance and barley yellow dwarf virus resistance (Kolmer *et al.,* 2008). Bolton *et al.,* (2008) speculated that Lr34 mediated resistance requires a high energy demand which leads to the stimulation of multiple metabolic pathways to support the cellular energy requirements.

This work was designed to study *in-silico* resistance genes which infer the possible function of rust resistance genes in wheat, through extensive literature search and data mining. In addition, their phylogenetic relationship was studied, and PCR primers were designed to identify rust resistance genes (durable and hypersensitive) in wheat

germplasm. Additionally molecular analysis of 16 local varieties to identify the Lr34/Yr18 gene complex, by using reported as well as specially designed PCR primers was carried out.

Materials and Methods

Sequence retrieval: Sequences of wheat rust resistance genes were retrieved through extensive literature search. Data mining was done on the public available databases *i.e*., NCBI Entrez, GrainGenes) and PlantGDB. After extensive review of reported and published entries of various resistance genes, only verified sequences were included in the study. These entries were examined for redundancy. Only after normalization of data; sequences were included for further study.

Alignment of the rust resistance genes to determine conserved sequence: Multiple sequence alignment of the rust resistance genes was performed to find the conserved sequence. Basic aim of this step was to find the sequence homology. ClustalW2 was used for this purpose. Amino acid sequences of the genes were retrieved from the NCBI Protein or translated via Expasy translation tool.

Motif finding: Motifs in the conserved sequence of resistance genes found via multiple sequence alignment were searched through 'Motif Search' and ScanProsite. The sequences which do not share any sequence homology with resistance gene sequences were also subjected to motif finding separately. After searching motifs in the sequences under study, extensive literature search was also conducted to determine the function of these motifs, hence to infer their possible role in the resistance.

Phylogenetic analysis: Phylogenetic analysis was determined to find out the origin and the relationship between resistance genes by using the 'A la Carte' mode of "Phylogeny.fr" program.

Primers designing: Primers were designed for MAS of resistance genes in the local wheat germplasm. Primer3 software with default parameters was used. These newly designed primers were also manually checked for GC content and self-annealing capability.

Seed germination of wheat varieties: Seeds of different wheat varieties (Jauhar-78, PIR-Sabak-04, Suleman-96, Seher-06, MARVI-2000, Fareed-06, Kiran-95, SIND-81, WC-21, WC-16, WC-1, LLR-32, LLR-9, WC-3, Lasani-08 and UR-0821), were germinated to get the leaf sample for DNA extraction for the molecular identification of Lr34/Yr18 gene complex in the wheat varieties. Seeds of the varieties were obtained from Wheat Research Institute, Faisalabad. The whole protocol from seed germination to plant growth is as follows:

Firstly filter paper was placed on petri-plate and water was poured on petri-plate. Seeds (5-6) were placed in each petri-plate and 20-25^{o}C temperature was provided. Seed germination occurred after 2-3 days. With the emergence of first leaf, germinated seeds were in condition to transfer to pots. Soil was taken from field and was ground by hands and then sieved. Mixed soil was prepared using 50% of sieve soil, 30% of compost and 20% sand. Pot hole was covered by a small stone and pot was filled with mixed soil. Seedlings were placed in such manner that caused least disturbance and damage to roots. Seedlings were watered with wash bottle to avoid any disturbance. Plant requirements were checked on daily basis.

DNA extraction: DNA extraction buffer was prepared as described by Nalini *et al*., (2004) and DNA was extracted with slight modifications in method described by Doyle & Doyle (1987). To verify the presence of DNA, samples were run on 1% agarose gel and then stored at 4^{o}C for further usage.

Polymerase chain reaction for detection of Lr34/Yr18 gene complex: Five sets of specially designed primers Gelenla 1, Renan 1, Renan3, CS1 and CS2 were used for detection of Lr34/Yr18 gene in the 16 local wheat varieties (Gelenlea 1 F5'TCGCTTCACTATTCGGCTTT3', R5'GTGTCGGAACACCCTAGCAT3'; Renan1 F5'CAAATTGTCTGCCACACCAC3', R5'ACAATGCCAAACACAGTGGA3; Renan3 F5'CATGTTCACCGGAGTTTGTG3', R5'AGCGAGAGGAGTCGATACCA3'; CS1 F5'TCAAGAACCTCCGCCTAGAA3', R5'TGATCGCCTAGACGCCTACT3'; CS2 F5'TAGGCGTCTAGGCGATCACT3', R5'ACTTCCAAATTCGACCATCG3').

Polymerase chain reaction was performed in 0.2 ml tubes (Axygen, USA) containing 20 µl total reaction mixture. The reaction was prepared by adding 2µl sample DNA, 2µl 10 X buffer, 2µl $MgCl_2$, 0.2µl dNTPs, 1µl of each forward and reverse primer (10µM), 0.2µl Taq DNA polymerase and 11.6µl PCR water. The reaction mixture was taken through thermocycling conditions consisting 5 min of 95°C for template DNA denaturation followed by 35 cycles of amplification each consisting of 3 steps: 45 sec at 94°C for DNA denaturation into single strands; annealing temperature in range of 50-63°C for 45 sec (depending upon the Tm of primers) and 45 sec at 72°C for extension of complementary DNA strands from each primer followed by final 7 min at 72°C for Taq DNA polymerase to synthesize any un-extended strands left. PCR was carried out in AB (Applied Biosystem) Veriti 96 well thermal cycler. Amplified products were run on 2% agarose gel.

Results

Sequence retrieval and multiple sequence alignment: After extensive literature search, a total of 12 leaf rust, 13 stripe and 2 stem rust resistance sequences were retrieved. These sequences were subjected to the multiple sequence alignment to determine sequence homology among these rust resistant genes. Among these studied sequences, 21 sequences confers hypersensitive response while remaining 4 are durable resistance genes. Hypersensitive response sequences were divided into four groups on the basis of sequence homology. First group contained hypersensitive induce response 3 (HIR3) and HIR4 proteins and their homology was found to be 55%.

Second group comprised of CIN14 and S11A11, which shared 98% homology. Kinases (Lrk10, Lrk19, Lrk33, RLK-R1, RLK-R2 and RLK-R3) formed third group and their percentage homology with each other was found to be in the range of 83%-95%. Fourth group contained sequences without any homology [Lr1, Lr10, Lr21, Yr5, Yr10, homeobox like resistance gene (HLRG), elicitor responsive gene 3 (ERG3) chitinase, glucanase and Xa21 like protein]. Wheat kinase Start domain protein (WKS1 and WKS2), Lr34 and Yr18 are durable resistance genes. WKS1 and WKS2 had 78% homology while Lr34 and YR18 shared homology greater than 95%.

Motif searching: Potential motifs found in genes with and without homology are listed in Table 1. Rest of genes shared no homology with each other, however they were also studied to identify motifs, and hence to assign their possible role in durable resistance or hypersensitive response.

Table 1. List of Motifs found in the Durable and Hypersensitive resistance Sequences.

Genes	Distinguished motifs	Genes	Distinguished Motifs
HIR3 and HIR4	SPFH domain/ band 7 family	Lr34 and Yr18	ATP-binding cassette, ABC transporter-type domain profile ABC-2 type transporter Plant PDR ABC transporter associated
Ag15, S11A11 and CI41N	NB-ARC domain LRR Disease resistance protein signature	Yr5	Disease resistance protein signature NB-ARC domain Leucine rich
Lr10	Disease resistance protein signature NB- ARC domain LRR Rhodopsin-like GPCR (G protein coupled receptors) superfamily signature	Putative stripe rust resistance like protein	Disease resistance protein signature Rhodopsin-like GPCR superfamily signature NB-ARC domain Leucine rich
Lr21	Cecropin family signature NBS-LRR LR21 rust-resistance class RGA Disease resistance protein signature NB-ARC domain LRR	Xa-21 like protein	Protein kinase domain Protein kinases ATP-binding region signature Serine/Threonine protein kinases active-site signature. Leucine rich repeat
Lr1	Disease resistance protein signature Bcl-2 apoptosis regulator protein family signature LRR NB-ARC domain	WKS1 and WKS2	START domain Cadherin signature Rhodopsin-like GPCR superfamily signature Protein kinase domain
Yr10	Resistance Stripe YR10 Rust NBS-LRR Disease resistance protein signature NB-ARC domain LRR Rhodopsin-like GPCR superfamily signature	Elicitor responsive gene 3 (ERG3)	C2 domain profile Cadherin signature Rhodopsin-like GPCR superfamily signature
Homeobox like resistance protein	Homeobox domain Homeodomain leucine zipper encoding Homez	Lrk10, rk19, Lrk33, LK-R1, RLK-R2 and LK-R3	Serine/Threonine protein kinases active-site signature Phosphotransferase family Protein tyrosine kinase Protein kinase domain
ChiA 0.1 gene andbeta-1,3-glucanase gene	Chitinase Class I Glycosyl hydrolases family 17		

Phylogenetic analysis: In the present study, majority of the genes belong to either NBS-LRR R gene family or kinases, so their phylogenetic trees were constructed to determine their relationship. Phylogenetic tree of the resistance kinases (Lrk10. Lrk19, Lrk33, RLK-R1, RLK-R2 and RLK-R3) is shown in Fig. 1. In the phylogram of kinases, there are two major clades. In first clade, Lrk10 and RLK-R3 forms a sister group and are more closely related. Second clade comprises of RLK-R1 and a possible unknown ancestor which has gone through further speciation in which Lrk33 and Lrk19 are closely related. RLK-R2 has formed an out-group.

Phylogenetic tree of the NBS-LRR members is divided into 2 main clades (Fig. 2). In first clade, Lr10 and Yr10 are closely related. Lr1 is related Lr10 and Yr10 with a possible unknown ancestor. In second clade, sequences S11A11 and CIN14 are shown to be similar. Putative resistance protein (prp), Yr5 and ag15 originated from a different ancestor, which is possibly more similar to ag15. Putative resistance protein ag15 and Yr5 form a sister group indicating that these are more closely related in second main clade; however, these originated from different ancestor.

Primers designing: Gene specific primers were designed (Table 2), which will help in the MAS. These primers were specifically designed for the identification of durable (LR34/YR18) and hypersensitive resistance genes in the local wheat varieties (kinases, NBS-LRR plant R family genes, WKS1).

Table 2. List of primers designed using primer3 for the detection of durable and hypersensitive rust resistance genes in wheat varieties.

Gene	Forward primer	Reverse primer	Product size (bp)
Hir3	GCTCCTACCTTTCCCCACTC	GGCTCAAGCACACTGTCAAA	161
	CCACTGCAAAGGATGTGATG	TAGCAAACCACAAACCACCA	236
	CGCTGTGAAACCAAGACAAA	GCGGACATAGCCTTCTCAAG	245
	GGCCATCTCACACTCAGGTT	TTCTGCACGAAAGCATCATC	230
Hir4	TGTCGCAAAGGCTGTACTTG	CCGCTTCTCCTTTGTAGACG	173
	TGTCGCAAAGGCTGTACTTG	GGATCTTCTCCGCTTCTCCT	182
	GTTCCAAGAATGGAGCTGGA	CCGCTTCTCCTTTGTAGACG	217
	TTCCAAGAATGGAGCTGGAC	CCGCTTCTCCTTTGTAGACG	216
Lrk33	GCCCAGAGCATGCTAACTTC	TTACTTGCCATAGCGCTCCT	229
	GCTGCTGATGTATTGCTGGA	TTACTTGCCATAGCGCTCCT	170
	AATAACATCAGCCCGTGGAG	GCCGGTAGATCAATCAGGAA	201
	TTGTCGGTTTTGATGTTCCA	ACGGCATTTAAGTCCTGGTG	198
Lrk10 and Lrk19	ATCCACCATGCCAATATCGT	TGGTTGCACCCTTGATGTAA	215
	TGGAACATCAAAACCGACAA	ACGATATTGGCATGGTGGAT	222
	AAAAAGTAGGGCAGGGAGGA	ACGATATTGGCATGGTGGAT	157
	GGCACGACGGTTTAAAGAAA	ACGATATTGGCATGGTGGAT	174
RLK-1, RLK-2, RLK-3	AGGCAAAATGGGCTACATTG	CCCATTGACCACTTTCTCGT	193
	AGGCAAAATGGGCTACATTG	TCCCCATTGACCACTTTCTC	195
	CTTCGACATCAAGCCTCACA	CACCATTTCTAGCACCAGCA	226
	CACTTCGACATCAAGCCTCA	CACCATTTCTAGCACCAGCA	228
S11A11 and CIN14	GACGACAGTTCGGGACAAAT	CAGGCATGGACCAATTCTTT	171
	AGCCATGCTGACACACAAAG	CTGAAAAGGCACTGGAAAGC	191
	GGCTGTTTTTCAAGGCATGT	ATTTGTCCCGAACTGTCGTC	183
	GGTGGGGCTAACACTTCAGA	TCCATCCACTCCTGTCCTTC	167
WKS1	GATTTTTCATCAGGCCTCCA	AGAAGCTGCAGTCCCACCTA	336
	CATCAGGCCTCCACATTTTT	AGAAGCTGCAGTCCCACCTA	359
	CGTGGCCAAAGGGTAGATTA	GCAATTAAAGGGGAGCATGA	347
	GATAGCAAATGCGAGCAACA	GAAGACGGAGGACCCATGTA	305
WKS2	TTGCTAACAAGCGTCCTCCT	CTGACTCGCCTTTTTGGAAG	240
	TTGCTAACAAGCGTCCTCCT	GTAACGGCAACACCACTCCT	168
	TTGCTAACAAGCGTCCTCCT	AGGTAACGGCAACACCACTC	170
	ATTCTTGCCATTTGCAGGTC	GGAGGCCTGAAGAAAAATCC	225
Lr34and Yr18	TCGCTTCACTATTCGGCTTT	GTGTCGGAACACCCTAGCAT	211
	ACGCTACGTCTTGGAGAGGA	TTGTCTTGCTCGATGTCAGG	228
	CACCAGCTCGAAACTGACA	CCACGTGATTATGACGCAAC	184
	CAAATTGTCTGCCACACCAC	ACAATGCCAAACACAGTGGA	159
	AACCGACAGTGACAGGAACC	CAGCTTGGTTTGGTTGGTTT	217
	CATGTTCACCGGAGTTTGTG	AGCGAGAGGAGTCGATACCA	177
	TCAAGAACCTCCGCCTAGAA	TGATCGCCTAGACGCCTACT	230
	TAGGCGTCTAGGCGATCACT	ACTTCCAAATTCGACCATCG	153
	TGCAGGTATGTGCCATGTTT	CAAGAGCTCGCAACAGAGTG	219
	ACCGGAAATGCTGAAACAAG	AAATGTTTCCTGCACCTTGG	214
	AGCATGCAGATTGGGAGACT	TGCGAGGTGACTTGAAACAG	186
	CCATGGAGCAGAGAAAGAGG	AATTTCAGTGCTCGGCTGAT	188

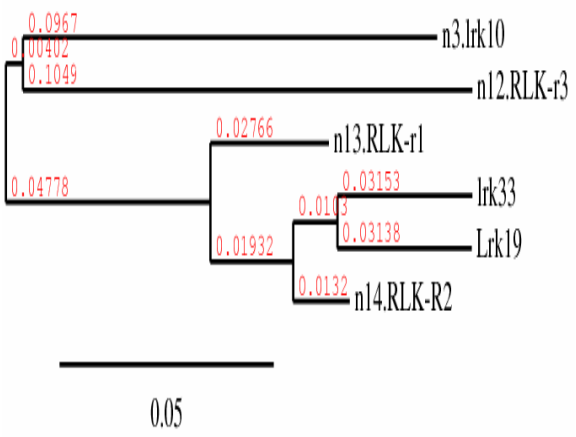

Fig. 1. Phylogram of leaf rust and stripe rust kinases.

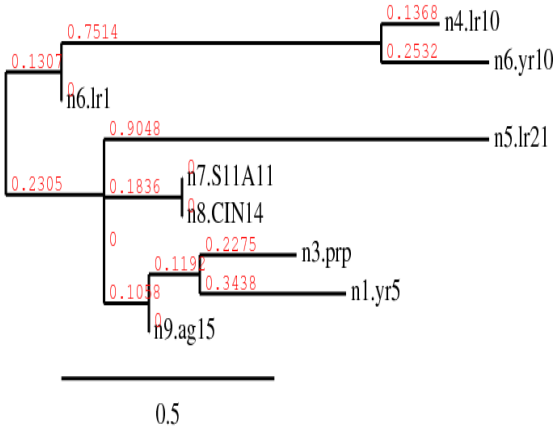

Fig. 2. Phylogram of NBS-LRR R proteins.

Molecular validation of rust resistance genes in local wheat varieties: PCR was carried out to detect durable rust resistance gene Lr34/Yr18 in local wheat varieties. For this purpose, five sets of primers designed in present study and one set of primers reported by Tiwari *et al.*, (2009) was used as reference. Self-designed primers yielded amplicon of correct length in all 16 wheat varieties studied. Renan 1 primer amplified 159 bp product specific for Lr34 at 63°C while Renan 3 primer amplified 177 bp products at 55°C. Glenlea 1 resulted in the amplification of 211 bp product specific for Lr34 at 57°C (Fig. 3a). CS 1 primer yielded an amplicon of 230 bp at 63°C while CS 2 primer amplicon was 153 bp at 63°C.

csLV34 primer was used to distinguish among LR34 positive verities, resistant and susceptible haplotypes on the basis of Lr34 allelic variation(Fig. 3b). Product size of the primer was 229 bp and 150 bp based on the amplification of allele a and b respectively. Allele 'a' containing varieties are susceptible while allele 'b' containing varieties are resistant. On the basis of PCR product size, studied varieties were found to have different rust resistance patterns, i.e. Jauhar-78, MARVI-2000, WC-1, LRR-32, LLLR-9 and WC-3 are resistant while remaining varieties are susceptible(PIR-Sabak-04, Suleman-96, Seher-06, Fareed-06, Kiran-95, SIND-81, WC-21, WC-16, Lasani-08 and UR-0821).

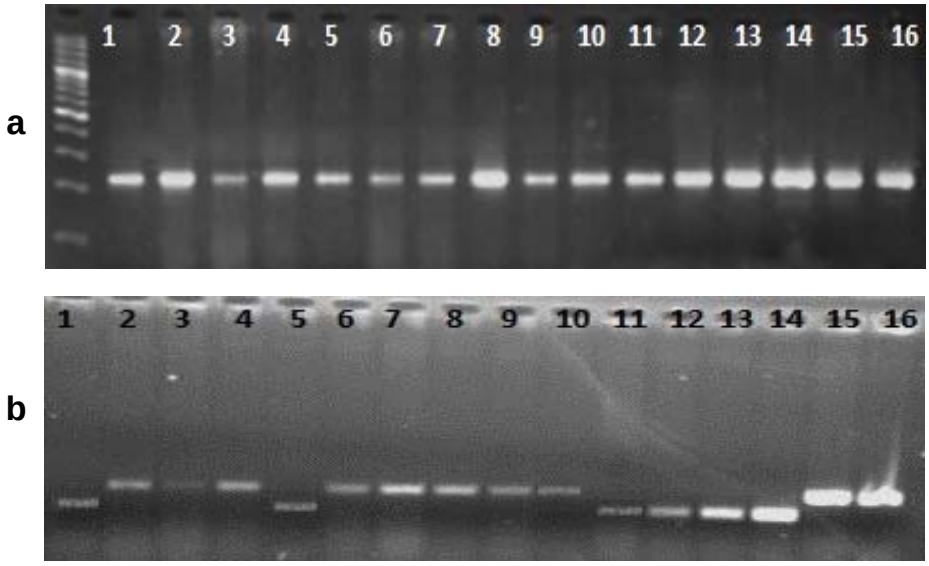

Fig. 3a. Identification of Lr34 locus in wheat varieties by gelenlea 1 primer (size of the amplicon is 211 bp) 3b. amplification of Lr34 gene by csLV34 primer, Lane 1, 5, 11, 12, 13 and 14 showing amplified 150 bp product of 'b' allele are resistant genotypes while remaining are susceptible varieties with amplification of 229 bp product 'a' allele . Lane 1-Jauhar-78; 2 Lane - Pir-Sabak-02; Lane 3-Suleman-06; Lane 4- seher-06; Lane 5-MARVI-2000; Lane 6- Fareed-06; Lane 7-kiarn-95; Lane 8-SIND-81; Lane 9-WC-21; Lane 10- WC-16; Lane 11- WC-1; Lane 12-LRR-32; Lane 13-LLLR-9; Lane 14- WC-3; Lane 15- Lasani-08; Lane 16-AUR-0821.

Discussion

Wheat cultivars are attacked by various types of rust pathogens and result in heavy yield losses. Wheat varieties become susceptible to rusts due to rapid pathogen evolution, and narrow genetic base for resistance (Babar *et al*., 2010). Genetic resistance is very important to mitigate yield losses and controlling epidemics (Khanzada *et al.*, 2011). Therefore, wheat production is mainly dependent upon the use and

development of resistant cultivars. In wheat, rust resistance genes can be classified into two categories, durable resistance genes and hypersensitive resistance genes (Hussain *et al*., 2011b).

In the present study, rust resistance gene sequences were retrieved and their alignment was carried out to find out the sequence similarity. On the basis of sequence homology studied, rust resistance genes/locus predominately belonged to either NBS-LRR gene family or kinase groups. It indicates that these genes are major players in generating similar resistance pathway. A large number of sequences (Lr1, Lr10, Lr21, Yr5, Yr10, Homeobox like resistance gene (HLRG), elicitor responsive gene 3 (ERG3) chitinase, glucanase and Xa21 like protein) do not form any group with specified function. Thus, it can be inferred that these genes might have role in generating defense response. Except for Lr34, Yr18 and WKS, which are durable resistance genes, rest of the genes under study were hypersensitive resistance genes.

Motif searching results showed that HIR3 and HIR4 proteins contained SPFH domain **(**Table 2). These motifs are involved in the regulation of ion channels in the plant pathogen response (HR) and in cellular proliferation (Zhang *et al*., 2011) . Ag15, S11A11, CI41N, Lr1, Lr10, Lr21, Yr5, putative stripe rust resistance-like protein and Yr10 are the part of nucleotide binding site-leucine rich repeat (NBS-LRR) gene family (El-aal *et al.*, 2008), which correlates with "Motif Search" output of the present work. NB-ARC domain is a nucleotide binding domain and LRR act as a pathogen effector or signal recognition molecule (DeYoung & Innes, 2006). Plant NBS-LRR proteins act through a network of signaling pathways to induce various defense responses including activation of oxidative burst, mitogen-associated protein kinase cascades, hypersensitive response and the induction of other genes related to pathogenesis (Mchale *et al*., 2006).

Another significant finding was presence of other motifs in these genes in addition to NBS-LRR domain i.e., rhodopsin like GPCR (G protein coupled receptors) signature motif in all studied genes containing NBS-LRR, cecropins family signature in Lr21 and Bcl-2 apoptosis regulator protein family signature in Lr1. These additional motifs might help in the downstream signaling of NBS-LRR through activation of oxidative burst by GPCR (Scheel, 1998; Tuteja, 2009), antifungal activity of cecropins (Haggag, 2008) and regulation of caspase activation by Bcl-2 regulators (Germain & Shore, 2003)**.**

Motif search results of the present study also showed that Lr34 gene and Yr18 gene contained ABC transporter. ABC transporter family uses the energy of ATP hydrolysis for performing diverse biological mechanisms (Krattinger *et al.*, 2011). Due to the presence of ABC transporter, it can be deduced that lr34 mediated resistance is by regulation of the transport of metabolites.

Xa21 like protein contained serine/threonine protein kinases active-site signature, LRR domain and kinase domain. Stpk are thought to involve in the early and downstream signal transduction to generate defense response i.e., mediating cell death and H_2O_2 production. LRR domain may involve in signal perception (Yang *et al.*, 1997; Cao *et al*., 2011). WKS1 and WKS2 contained protein kinase domain as well as START domain. Initially Yr36 was identified but later on WKS1 was verified due to same function was verified to be the same gene. The kinase START domain confers temperature dependent resistance against stripe rust (Fu *et al*., 2009).

Stripe rust and leaf rust kinases (Lrk10, Lrk19, Lrk33, RLK-R1, RLK-R2 and RLK-R3) were found to have protein kinase domain with serine/threonine protein kinases active-site signature in the present study. All of RLK are receptor kinases, have an extracellular domain for ligand binding, a trans-membrane domain spanning the cell membrane and cytoplasmic kinase domain for downstream signaling (Song *et al*., 1995; Shiu & Bleecker, 2001).

In the present study, majority of the genes belong to either NBS-LRR R gene family or kinases. Phylogram of kinases depicts that there are two major clades. In first clade, Lrk10 and RLK-R3 forms a sister group and are more closely related. Greater branch length of RLK-R3 depict that it is more distant. Second clade comprises of RLK-R1 and a possible unknown ancestor which has gone through further speciation in which Lrk33 and Lrk19 are closely related. This could be due to the emergence of these resistance genes at the same time, but they show divergence in their functionality. RLK-R2 forms an out-group, and its smaller branch length depicts that it is relatively recent. Phylogram of NBS-LRR members divided into two main clades. In first clade, Lr10 and Yr10 are closely related. Yr10 has greater amount of evolutionary time. Lr1 is similar to the possible unknown ancestor. In second clade sequences S11A11 and CIN14 are shown to be similar. Ag15 is believed to be an exotic candidate for Lr19 mediated resistance (Gennaro *et al.*, 2009) but it is more recent than former two. Putative resistance protein and Yr5 form a sister group and are more closely related in second main clade.

Identification and designing of new PCR primers are needed to select rust resistant germplasm from wheat breeding stocks in early segregating generations. In order to detect durable rust resistance and hypersensitive resistance, there is a constant need to device selection markers. As Lr34, Yr18 and Yr36, kinases and NBS-LRR were found to be dominantly involved in the HR and durable resistance, a set of 44 primers were designed. According to finding of this work, identification of the NBS-LRR resistance genes and kinases using these molecular markers will serve be valuable for selection of rust resistance genotypes from breeding stocks. Among these, a set of 20 primers are specifically designed for durable rust resistance genes detection (Lr34, Yr18 and Yr36).

Further, 5 sets of newly designed primers were used to validate presence of Lr34/yr18 locus in 16 local wheat varieties. Lr34/Yr18 is very important and valuable locus. It is a multiple pathogen resistance locus i.e., it confers resistance against leaf rust, stripe rust, powdery mildew and barley yellow dwarf virus. These primers confirmed the presence of Lr34 locus in all local wheat varieties under study. However, presence of Lr34 does not guarantee presence of durable rust resistance; diagnostic markers were used to differentiate between resistant and susceptible genotypes. A sequence tagged site marker csLV34 (Tiwari *et al*., 2009) was used to differentiate

between resistant and susceptible varieties. Jauhar-78 was used as positive control in the present study, as it is already reported to have csLV34b (resistant) allele (Kolmer *et al*., 2008). Molecular validation of Lr34 gene in other studied varieties was not found to be reported in authentic literature. Therefore, in this study, for the first time these local varieties were validated for the presence of Lr34 gene. Jauhar-78, MARVI-2000, WC-1, LRR-9 and WC-3 contained 150bp amplicon and are believed to be resistant while remaining are susceptible.

It can be concluded that these newly designed markers are viable diagnostic markers for the detection of Lr34 and identification of +Lr34/-Lr34 haplotypes in local wheat varieties. However, kinases and NBS-LRR detection is also needed to identify resistant genotype. Breeders can use these Lr34 possessing varieties in cultivation, and to rapidly incorporate Lr34/Yr18 or 'multiple pathogen resistance locus' in other wheat varieties to mitigate the yield losses caused by leaf rust epidemics. Researchers can also combine other durable resistance genes with Lr34 and make it the 'invincible gene'.

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