Cloning and analysis of NBS-LRR super family of resistance (R) genes in wheat (Triticum aestivum L.)

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Abstract: Resistance (R) genes containing nucleotide binding site (NBS) and leucine rich repeats (LRR) are the most prevalent types of resistance (R) genes in plants. The objective of this study was to isolate, identify and analyze resistance genes from disease (rust) and resistant wheat lines by PCR based strategy. Fifteen degenerate primers were designed from the conserved kinase-la and hydrophobic domains of known NBS-LRR type R-genes and from EST data bases. Four advanced resistant lines and one susceptible wheat line was selected from the trap nursery. Out of hundred primer combinations only seventy five primer combinations showed amplification. Twenty two primer combination showed differential banding pattern which were not present in highly susceptible Morocco, were cloned in TA based cloning vector and got them sequenced. Sizes of sequenced nucleotides were between 500bp to 1500bp. The cloned fragments showed their DNA sequence similarity to known resistance (R) genes of NBS-LRR family. These results indicate that identified genes are the valuable source to use as disease resistance genes or to screen wheat resistant germplasm against different types of rusts.

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Introduction

Wheat (*Triticum aestivum*) is among the most widely grown cereals, occupying 17% of the total arable land in the world. About 35% of the world's population utilizes wheat as the major staple food (CIMMYT, 2006). However, yield of this crop is susceptible to a number of biotic and abiotic stresses. Of various biotic stresses, attack of fungal diseases is the major determinant of wheat yield worldwide. Rust attack on wheat falls in three categories, stem rust caused by *Puccinia graminis*, leaf rust by *Puccinia triticina* and stripe rust by *Puccinia striiformis*. There exists variation in specific infection type that greatly fluctuates among wheat cultivars, rust races and temperature. However, infection type could be highlighted by determining the size and coloration of rust pustules (McIntosh *et al.,* 1995b).

The most common type of leaf rust is caused by *Puccinia triticina* Erikss which has become a serious threat in wheat growing countries (Knott, 1989). The reduction in yield due to rust disease could reach 62% under favorable conditions. The vulnerability to rust becomes higher when homogenous wheat cultivars are grown over the large area. The resistance to rust is greatly determined by single race-specific resistance genes in genetically homogenous wheat cultivars (Samborski, 1985). Rust causing fungus has undergone a number of mutations that led to the development of virulent races. Therefore, there is a continuous need to breed wheat plants for rust resistance so as to combat the newly evolved virulent rust fungus (Kilpatrick, 1975).

Wheat varieties under cultivation are sensitive to different pathogens including fungi, bacteria and viruses. Diseases due to these pathogens result in greater yield losses in wheat. There has been extensive research to improve the resistance in wheat against important diseases. However, inefficiency of selected procedures is the major barrier in improving plant tolerance to lethal diseases. Resistant (R) genes from different model plants have been cloned and these genes are reported to encode important components of signal transduction (Baker *et al.,* 1997).Therefore, race-specific resistance is not given much importance since it is derived from the deployment of single genes. However, resistance could be enhanced by pyramiding different efficient race-specific genes into one cultivar. This will induce pathogen to undergo a number of mutations that reduces the chances of infection (McDonald and Linde, 2002).

There are five different classes of R genes sharing common structural domains of proteins. Resistant (R) genes encode proteins having leucine-rich repeat (LRR) region, nucleotide binding site

(NBS), kinases (intracellular serine/threonine kinases), extracellular LRR proteins with a short cytoplasmic domain and a transmembrane (TM) domain, RPW8 class of protein having a tansmembrane domain and coiled-coil (CC) domain and extracellular LRR (leucine-rich repeat) protein containing transmembrane domain and intracellular kinase domain (Dangl and Jones, 2001).

Resistant (R) genes encoding NBS-LRR proteins constitute the largest group of R genes. This class of R genes is more prevalent among plants. For instance, presence of more than 150 and 160 putative NBS-LRR genes have been reported in *Arabidopsis* (Arabidopsis Genome Initiative, 2000) and rice (Goff *et al.,* 2002) genomes. Moreover, R genes exist in clusters with paralogs of R genes (Young, 2000). Sequence analysis of R genes could not predict cellular localization of protein products. Generally, these are cytoplasmic proteins and only one protein RMP1 is thought to be associated with the plasma membrane (Boyes *et al.,* 1998). There exist two groups of NBS-LRR proteins differing in their amino-terminal sequence. First group possesses TIR domain which is homologous to mammalian interleukin-1 receptor (IL-1R) and intracellular signaling domain of the Drosophila Toll. These proteins are referred to as TIR NBS-LRRs (Whitham *et al.,* 1994). The second class of NBS-LRR proteins is known as CC-NBS-LRRs which have CC domain at the amino-terminus (Bent *et al.,* 1994; Dangl and Jones, 2001).

RLPs (receptor-like proteins) represent second class of R genes with an amino-terminal extracellular LRR domain, carboxyl terminal cytoplasmic domain and transmembrane domain (Meyers *et al.,* 2005). In this context, Jones et al. (1994) reported the resistance to the fungus *Cladosporium fulvum* due to Cf genes in tomato. HcrVf2 gene from apple (Belfanti *et al.,* 2004) and RPP27 gene from arabidopsis (Tor *et al.,* 2004) also belong to this class of R genes.

Most classes of R proteins have LRR domain. This domain is characterized with varying number of repeated motifs with leucines or hydrophilic amino acids at specific distances (Jones and Jones, 1997; Martin *et al.,* 2003). Furthermore, a diversity of proteins performing a variety of functions are known to possess LRR domains which are involved in protein-carbohydrate or protein-protein interactions, and peptide ligand binding (Kajava, 1998). LRR domains of proteins play a crucial role in recognition specificity (Ellis *et al.,* 1999; He *et al.,* 2000; Dodds *et al.,* 2001). Better tolerance to base substitutions is present in R genes with LRR domains. Base substitutions are important evolutionary changes exhibited by pathogens (Dinesh-Kumar *et al.,* 2000; Axtell *et al.,* 2001; Tornero *et al.,* 2002). Moreover, LRR proteins are also known to be involved in signaling (Warren *et al.,* 1998; Hwang *et al.,* 2000). In addition, exact nature of NBS domain is not known. However, NBS domain is composed of about 300 amino acids followed by amino-terminus region of NBS-LRR proteins and NBS sequences. There are conserved amino-acid motifs in NBS domain (Meyers *et al.,* 1999; Meyers *et al.,* 2003). Research on resistance based on R genes is among the crucial approaches that impart tolerance to wheat against rust. Therefore, the objective of this study was to isolate, identify and analyze resistance genes from disease (rust) and resistant wheat lines by PCR based strategy.

Material and Method

Identification of rust resistant and susceptible genotypes

For present studies four local resistant wheat lines and one susceptible line was selected after surveying Wheat Research Institute (WRI), Ayub Agricultural Research Institute (AARI) Faisalabad. These lines were

1. E-13 V-87094/2* PAK-81//PBW-343 Resistant
2. E-14 V-87094/2* PAK-81//CH-2000 II
3. E-16 V-87094/2* PAK-81//CI1-2000 II
4. E-37 V-87094/2* INQ-91 //YOCORA-73 II
5. MOROCCO Susceptible

Selected Wheat lines (planted at WRI and AARI) were sprayed with rust inoculums to check and confirm the resistant and susceptible lines of wheat in a trap nursery and let the disease spread. Leaf samples from each selected line were collected and kept at -80 ^{o}C freezer until DNA extraction. All subsequent work was done at Wheat Biotechnology Lab at NIBGE. Total DNA was isolated from leaf tissue by using standard CTAB method and Genomic DNA Purification kit of Fermentas Life Sciences. Intact DNA was used as template in subsequent PCR amplification.

Degenerate primers were designed from highly conserved motifs, kinase-1a (K) and hydrophobic domain (HD) of NBS-LRR type resistance genes. NBS-LRR sequences were obtained from known R-genes and EST databases of monocots in order to target the gene family. The following fifteen degenerate primer pairs were designed and used in all possible combinations for amplifications.

PCR was performed by using various combinations of degenerate primers designed from highly conserved motif of NBS-LRR super family of resistance genes. PCR was carried out in a final reaction volume of 25µl containing 2.5µl (25ng/ µl) of DNA, 2.5 µl of 10x PCR buffer, 2µl MgCl2 (25mM), 0.5µl of dNTPs (10mM each) 0.5 µl of degenerate primer (100ng/µl) as forward primer, 0.5µl of second degenerate primer (100ng/µl) as reverse primer, 0.2µl

of *Taq* DNA Polymerase (5U/µl; Fermentas, USA), 16.3µl double distilled PCR grade water. The PCR amplification profile include initial cycle of denaturation at 94^0C for 1 min and 10 sec; 94^0C for 10 sec; 60^0C for 45 sec; 72^0C for 1min 10sec for 5 cycles followed by 38 cycles at 91^0C for 10sec; 55^0C for 1min; 72^0C for 1 min 10sec with final extension of 4min 30sec. MJ Mini^{TM} Personal Thermal Cycler (BIO-RAD, USA) was used for the PCR amplification. The amplified products were resolved on 1% agarose gel and stained with ethidium bromide.

Table 1. DNA sequences, melting temperatures (T_m) and degree of degeneracy of degenerate primers.

Primer	Sequence (DNA)	T_m (°C)	Degeneracy*
K01	5'GGSGGGGTGGGGAAGACSAC3'	65.6	4
K02	5'GGWGGGGTTGGGAAGACWAC3'	58.3	4
K03	5'GGSGGSGTGGGTAARACDAC3'	60.9	24
K04	5'GGTGGCGTGGGCAAGACDAC3'	62.8	3
K05	5'GGGGGSATGGGYAARACDAC3'	59.9	24
HD01	5'GAGGGCGAGGGGGAGGCC3'	65.7	0
HD02	5'CCAACGCCAATGGAAGACC3'	57.3	0
HD03	5'AAGNCTAARGGGAGGGCC3'	57.1	8
HD04	5'GAGCGCCARCGGGAGGCC3'	65.8	2
HD05	5' GAGVGCGAAGGGGAGGCC3'	62.6	3
HD06	5'GAG VGC CAR CGG NGA GCC3'	63.3	24
HD07	5'GAG VGC CAR SGG RTG GCC 3'	63.4	24
HD08	5'GAG VGC CAR SGG YTT GCC 3'	61.6	24
HD09	5'GAG VGC CAR SGG RTT GCC 3'	61.2	24
HD10	5'HTA VGC CAR KGG RTT GCC 3'	56.6	72

*Degree of degeneracy of primer sequences with degenerate nucleic acid bases: R=A,G; Y=C,T; K=G,T; S=C,G; W=A,T; H=A,C,T; V=A,C,G; D=A,G,T; N=A,C,G,T.

The desired differentially expressed DNA fragments were eluted from agarose gel. The elution was done by using Jena Bioscience Agarose Gel extraction kit (Jena Bioscience, Germany). The differentially amplified DNA fragments were cloned into pTZ57R/TA cloning vector by using PCR cloning kit (Fermentas Life Sciences). The cloning site in TA is within the *LacZ* gene which helps to easily identify transformants containing desired gene on the basis of insertional inactivation. The transformed plasmid DNA was digested with *XbaI* and *HindIII* enzyme at 37ºC for 2- 3 hours. The samples having the correct inserted fragments were selected. Clones were sequenced from Macrogen, Korea according to standard Sanger Sequencing protocol. Each insert of the appropriate size was sequenced by both M13 forward and reverse primer. DNA sequence data was blast searched by exploiting different tools of NCBI. The translation of the DNA sequences was done by utilizing Expasy's translation tool. Sequence alignment was done with CLUSTALW and ALIGN on the Expasy website according to Pearson *et al*. (1997). A motif and pattern scan was performed on the amino acid sequences according to Falquet *et al*. (2002) on the PROSITE database.

Multiple Expectation Maximization for Motif Elicitation or 'MEME' (Bailey and Elkan, 1995), was used to analyze conserved motif structures among NBS sequences. MEME discovers motifs by using a statistical algorithm called expectation maximization in unaligned sequences with no *a priori* assumptions about the sequences or their alignments. MEME reports a profile that describes a mathematical pattern in the conserved sequences. An individual profile describing amino acid frequencies is generated for each motif. Each position in the profile describes the probability of observing each amino acid at that position. Matches between the profile and individual sequences are scored by the program for each amino acid along the width of the profile. Multiple MEME analysis was performed with settings designed to identify 20, 30, 40 and 50 motifs; increasing the number of motifs simultaneously separates related motifs in different class sequences. The program MAST (Bailey and Gribskov, 1998) was used to assess correlations between MEME motifs in the distance matrix.

Results

Four local resistant wheat (*Triticum aestivum*) lines (E13, E14, E16 and E37) and one susceptible variety (Morocco) was included in this study. Morocco being highly susceptible to all types of rusts, was used as marker. Total genomic DNA was isolated to use as template in subsequent PCR procedure (Figure 1).

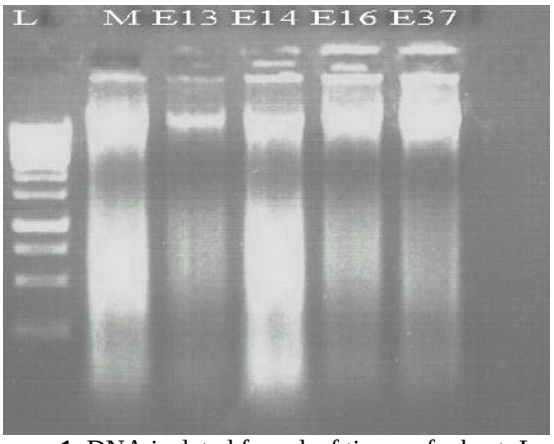

Figure 1. DNA isolated from leaf tissue of wheat. Lane 1 represent 1kb DNA ladder, Lane 2 represent M=Morocco (highly rusts susceptible wheat variety), Lane 3 to 6 represent E13, E14, E16 and E37 (highly rusts resistant wheat lines).

To identify the resistance R genes in resistant lines of wheat which were not present in Morocco, 100 primer combinations (in all possible combination e.g. KO1 * HD01, HDO1 * HD10) were used. Out of hundred primer combinations, only seventy five primer combinations gave amplification. Twenty two primer combinations showed differentials which were not present in highly susceptible Morocco (Figure 2).

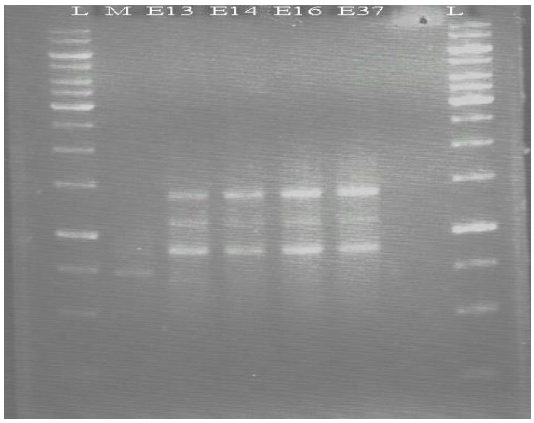

Figure 2. Representative PCR amplification profiles from 1% agarose gel stained by ethidium bromide and visualized under UV light depicting 1400 bps and 1100 bps fragments. Lane 1 and 8 represent 1kb DNA ladder, Lane 2 represent M=Morocco (highly rusts susceptible wheat variety), Lane 3 to 6 represent E13, E14, E16 and E37 highly rusts resistant wheat lines, Lane 7 is empty.

Differentially amplified genes were cloned in TA vector. Plasmid DNA was isolate and digested using restriction enzymes (*XbaI* and *HindIII*). Clones which produced identical insert sizes and restriction fragment were considered to be products of a single amplification event. No additional clones were sequenced. Therefore, the plasmids to be sequenced were selected based on the result of their restriction digestion. A total of 15 plasmids that showed desired sizes were sequenced (Table 2).

Table 2. Clones chosen for sequencing

No	Gene Name	Gene Size (bps)	Source (wheat Lines)	Primer Pair
1	**NL2C**	1100	E13	HD02, HD03
2	**NL3C**	1250	E14	HD02, HD03
3	**NL4B**	750	E13, E14, E16 & E37	HD02, HD05
4	**NL9C**	1500	E13	KO4, HD02
5	**NL15F**	600	E13, E14, E16 & E37	K04, HD03
6	**NL16A**	750	E13, E14, E16 & E37	K04, HD03
7	**NL18A**	1500	E13	K03, HD05
8	**NL23B**	600	E13 & E37	K04, HD08
9	**NL24C**	300	E13 & E37	K04, HD08
10	**NL26B**	800	E16 & E37	K01, HD10
11	**NL27C**	500	E13, E14 & E37	K03, HD10
12	**NL30F**	1000	E13, E14, E16 & E37	HD06, HD07
13	**NL37A**	900	E13, E14, E16 & E37	K01, HD06
14	**NL37C**	750	E13, E14, E16 & E37	K01, HD06
15	**NL38A**	1500	E13, E14, E16 & E37	K01, HD06

Sequences were first exposed to the VecScreen algorithm in order to remove the vector contamination. The insert sequences were then compared to the nucleotide and protein sequences available at the Entrez nucleotide and protein databases using the BLASTN, BLASTX, and TBLASTX algorithms. Out of the 15 sequences, 5 did not show significant homology to any known sequence, 6 were significantly

similar to genes that were not related to disease resistance, and 4 were homologous to known R genes. Table 3 shows a list of 9 clones that were matched with different accessions in the gene bank (Table 3).

Table 3. Accessions match by blast

No	Gene Name	Accessions match by blast
1	NL2C	AY951944.1, AM932686.1, AB298185.1
2	NL4B	AM932684.1
3	NL9C	K01229.1
4	NL15F	DQ537335.1, AY494981.1, EF081025.1
5	NL16A*	AM932680.1, AY485644.1, AF446141.1
6	NL18A	EF664750.1, DQ490951.2
7	NL23B	EU660891.1
8	NL24C	AM463372.2
9	NL30F	AY368673.1

Discussion

Major yield losses in wheat are often associated with rust diseases (Roelfs *et al.,* 1992). Rust causing fungus is under continuous mutation resulting in the generation of new fungal races that have the potential to break the resistance of existence varieties. Susceptibility of wheat plants to rust increase due to different reasons including little variation in genome, cultivation of one variety over a long area, introduced pathogen and failure of varietal resistance. Different race specific leaf rust resistance genes (Lr21, Lr1 and Lr1O) have been cloned (Feuillet *et al.,* 2003; Ling *et al.,* 2002; Huang *et al.,* 2003). However, most of Lr genes are ineffective (Long et al, 1988: Ayliffe and Lagudah, 2004). R genes do not provide wide spectrum resistance due to recognition of limited number of pathogen races. In addition, pathogen exhibit adaptation to this resistance over time (Ayliffe and Lagudah, 2004). To avoid the spread of disease, new wheat cultivars must be released continuously.

In the literature, only two studies were found that directly aimed to amplify RGAs from a cDNA template. Liu and Ekramoddoullah (2003) reported the amplification of RGAs from cDNA of western white pines that have been inoculated with resistant to the white pine blister rust (*Cronartium ribicola*). This method is very valuable for the purpose of identifying R genes conferring resistance to a specific pathogen. However, in this study no such specific pathogen was in question. Therefore, the plants were not inoculated with a certain pathogen. Budak *et al.* (2006) reported the amplification of RGAs from cDNA of buffalo grass grown in soil, without any pathogen application. Soil and open air always contain a wide spectrum of microorganisms, so the RGAs they have reported may be R genes expressed in response to these pathogens or those that are constitutively expressed. In this study, constitutively expressed RGAs were targeted, since they are likely to be functional R genes. Therefore, the plants were grown in a pathogen (rust) environment. To our knowledge, this study is the first to isolate RGAs from DNA of wheat plants grown under pathogen environment.

It is known that some R genes are constitutively expressed in plants, but at very low levels (Michelmore and Meyers, 1998). In previous studies, the amplification of genomic DNA or cDNA of various plants with primers targeting the kinase 1a and GLPL motifs has almost always resulted in clear bands of the expected size (500 bps) in gel electrophoresis analyses, usually along with clear bands of different sizes (Leister *et al.,* 1996; Deng *et al.,* 2000; Di Gaspero and Cipriani, 2002; He *et al.,* 2004; Budak *et al.,* 2006). We used a PCR-based approach with degenerate primer combinations that targeted the conserved domains K (GVGKTT) and HD (GLPLAL) of the NBS-LRR class of plant. In this study a clear and bright band of 500 bps was visible (Figure 3.3) but not eluted because this size of bands also present in Morocco, the susceptible wheat line. So, all of the eluted bands of different sizes mention in Table 3.1 were not present in Morocco to ensure the resistant because all the disease (rust) genes present in Morocco were susceptible to disease (rusts). The aim of this study was to isolate disease resistant genes which were not present in susceptible wheat (*Triticum aestivum*) varieties grown in Pakistan. Morocco was used as check line to isolate potential disease (rusts) resistant genes from wheat.

In this study, the yield of RGAs recovered was very low. Out of 40 colones screened, 15 were sequenced, Out of the 15, 5 did not show significant homology to any known sequence, 6 were significantly similar to genes that were not related to disease resistance, only four of which gave similarity hits to known R genes. This might be a result of the low success of the RGL primers used to amplify degenerate fragments. Most of the clones sequenced were homologous to unknown sequences or genes unrelated to disease resistance.

The present studies concluded that degenerate primers from conserved domains may be further utilized for mapping of other gene families, provided that they have enough conservation in two or three domains within their protein sequence. It is the sequence information and better sequence analysis tools that make this approach applicable for ‘gene family targeted mapping via PCR. As shown in this study, both amino acid and codon usage diff erences at the priming sites can be utilized in designing degenerate primers, which would target all possible amino acid and codon diff erences of a given conserved

domain in a gene family. Such primers would target all genes carrying any combinations of those domains known to exist in that particular gene family. Indeed, a similar approach has been used to study the peroxidase gene family in grasses.

The present studies indicate that identified genes are the valuable source to use as disease resistant genes for the improvement of susceptible wheat (*Triticum aestivum*) varieties. Full length sequences of these genes will be cloned and used for developing rust resistant wheat (*Triticum aestivum*).

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