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Full Length Article

Molecular Identification of Stem Rust Resistance Gene(s) from Pakistani Wheat Cultivars

Shahzad Bashir[1], Shazia Anwer Bukhari[1*], Irfan Afzal[2], Ghulam Mustafa[1] and Mahmood-ur-Rahman[3]
[1]*Department of Biochemistry, Government College University, Faisalabad-38060, Pakistan*
[2]*Department of Agronomy, University of Agriculture, Faisalabad-38040, Pakistan*
[3]*Department of Bioinformatics and Biotechnology, Government College University, Faisalabad-38060, Pakistan*
[*]For correspondence: shaziabukhari@gcuf.edu.pk

Abstract

Wheat is a major part of our diet and 90% of this crop is facing threat of stem rust disease worldwide, which is caused by *Puccinia graminis* f. spp. *tritici* (*Pgt*). In Pakistan, most of the wheat cultivars are susceptible to the disease and very little evidence based data is available about stem rust resistant cultivars. Predictability of *Pgt* migration from Africa to Pakistan through Iran makes this issue important so that its molecular surveillance is very important to improve our wheat genetically. This study is based on screening of *Sr* gene family and some other genes (*RPG* genes) against Pakistani wheat germplasm. Total 108 wheat cultivars were screened for different reported resistant genes. *Sr22* gene was selected for further analysis and isolated and cloned. *Sr22* was triggered by inducing *P. graminis* on healthy resistant cultivars such as Mexi-Pak and Auqab 2000. After induction total mRNA was isolated and used to synthesize cDNA for the isolation of full-length gene. It was ligated in pUC57 vector and sequenced. The sequence comparison indicated a slight change in nucleotide sequence but amino acid sequences were found to be identical. Screening data and transgenic plants containing *Sr22* gene isolated in this study will be shared with wheat breeders to tailor new varieties having better resistance against stem rust. © 2019 Friends Science Publishers

Keywords: Auqab 2000; Mexi-Pak; *Puccinia graminis*; *RPG* genes; *Sr22*

Introduction

Wheat (*Triticum aestivum* L.) is not only the major staple food for the people of Pakistan but also the most important cereal crop in the world. It is the main ingredient of human food and has been feeding humans since its domestication by 10,000 to 15,000 BC. In the current era, 40% of the world's population is feeding on wheat which contributes 20% in terms of total proteins and calories intake (Gupta *et al*., 2008).

Wheat is among the major crops of Pakistan and is also an important part of diet of about 90% Pakistani population. Wheat has been considered as the backbone of the economy of Pakistan as its share is about 10.3% in agriculture and 2.2% in country's GDP (Usman, 2016). In Pakistan, wheat is cultivated on 9.20 Mha with an average yield of 2.66 tones/ha (Ali *et al*., 2017). Wheat stem rust can cause a great damage to wheat production in Pakistan that will strongly disrupt country's economy (Sheikh *et al*., 2017). Therefore, scientists have to develop resistant cultivars and also screen out germplasm that would be used in a breeding system for resistant variety production in future.

In history, *Puccinia graminis* distorted wheat crop many times and caused thousands of deaths through starvation. It destroys 50 to 70% crop on a large area but individual fields can be totally destroyed (Singh *et al.*, 2008). The losses are more severe when disease appears before the grain set because of damage to the stem causes reduction of nutrients and food supply to the grains. This results in shrunk and low-quality seeds. Some other diseases and logging due to *P. graminis* add more difficulties and loss until harvesting (Singh *et al.*, 2011).

P. graminis produces uredinia on the surface of the stem which are the main indication of the presence of stem rust. Uredinia are brick red, elongated and blister-like pustules that are easily shaken off. Commonly they are present on leaf sheaths but can also be observed on the stem, leaves, awns and glumes (Singh *et al.*, 2008). They are developed mostly underside of leaf but can also appear on the upper side of the leaves as well. On leaf sheaths and glumes pustules rupture the epidermis and give a ragged appearance (Szabo *et al.*, 2014).

In 1999 a new strain appeared in Uganda named as Ug99 (*P. graminis* f. spp. *tritici*) that broke the resistance of existing wheat cultivars having *Sr6, Sr7a, Sr7b, Sr9a, Sr9b, Sr10, Sr11, Sr12, Sr16, Sr17* and *Wld1*genes (Singh *et al.*, 2008). In North America about 16% of hard red spring

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wheat, 28% soft winter wheat and 48% hard winter wheat showed resistance against the Ug99 (Jin and Singh, 2006).

Different methods and techniques are used to control diseases such as host resistance use of pesticide, biological control using natural enemies and using resistant cultivars. Resistant cultivars perform excellently by providing resistance against stem rust over half a century but then Ug99 appeared which broke the resistance (Jin *et al.,* 2007). Among 56 designated and a few undesignated stem rust resistance genes in wheat, only eight designated genes in the primary gene pool (*i.e.*, *Sr13, Sr14, Sr22, Sr28, Sr33, Sr35, Sr42* and *Sr45*) confer resistance to *P. graminis* f. spp. *tritici* (Pretorius *et al.,* 2000; Jin *et al.,* 2007). It should be noted that Norin 40 was used as tester line for *Sr42* and it was assumed that the resistance of Norin 40 to *P. graminis* f. spp. *tritici* was due to *Sr42*. *Sr42* initially designated as *Sr*-Norin 40, was derived from winter wheat cultivar Norin 40 located on the short arm of chromosome 6D (McIntosh *et al.,* 1995).

Sr13, Sr14, Sr22, Sr28, Sr33, Sr35, Sr42, and *Sr45* can be used by advanced biotechnological tools to make resistant cultivars. These genes were lost during evolution from diploid to hexaploid and some cultivars which showed the presence of these genes did not produce enough expression to confirm resistance.

The resistance against *P. graminis* decreases as the breeding pressure was shifted towards production from quality. The genes that confirmed resistance were lost during the breeding of new high yielding cultivars. Some cultivars have resistance genes in their genome but their expression level is very low. The low expression is due to climate change mainly due to high temperature. Stem rust and ribosomal protein genes such as *Sr22, Sr33, Sr35, Sr45, RPG1, RPG4* and *RPG5* have not reported in hexaploid wheat. Therefore, this study was aimed to isolate potential gene(s) that confirm resistance against *P. graminis* from hexaploid wheat of Pakistani origin.

Materials and Methods

Plant Material

The locally available commercial 58 wheat cultivars were collected from Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan (NIAB) along with 50 breeding lines from University of Agriculture, Faisalabad, Pakistan. The collected 108 wheat cultivars were grown in a growth chamber. These plants were allowed to grow for 2 weeks at 20/16ºC day/night temperature.

DNA Isolation and PCR

When the wheat plants started to grow, they were taken for DNA extraction using CTAB method (Borges *et al.,* 2009). The DNA sequences of selected genes were retrieved from GenBank and primers were designed for the confirmation of these genes in germplasm collections. Primers were designed with the help of bioinformatics tool SnapGene (Huep *et al.,* 2014) and given in Table 1. PCR amplification was carried out to screen 108 cultivars against selected genes with the help of gene-specific primers. PCR program used was: denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension for 1 min at 72°C. The verities that showed very bright bands were selected for the isolation of full-length genes (*e.g.*, AS2002, Mexi-Pak and Auqab 2000).

Gene Frequency in Germplasm

Presence of rust resistant genes in germplasm was measured in percentage. It was calculated by dividing the total number of cultivars in which gene is present by total number of cultivars and multiplied by 100 (Staub, 1998).

$$\text{Gene frequency in \%} = \frac{\text{No. of cultivars in which gene is present}}{\text{Total No. of cultivars}} \times 100$$

Full-length Gene Isolation

Sr22 was selected for full-length gene isolation due to its highest level of stable resistance (Steuernagel *et al*., 2016). Mexi-Pak and Auqab 2000 were grown in growth chamber and induced by spraying inoculant of fungus. *P. graminis* was obtained from Institute of Plant Pathology, Ayub Agriculture Research Institute, Faisalabad, Pakistan. After 24 to 36 h of incubation the solution was sprayed on healthy plants and placed in glass house in a separate chamber. After 72 h of inoculation, RNA was isolated from young leaves using Trizol method (Sah *et al.*, 2014). cDNA was synthesized by Revert Aid H minus cDNA synthesis kit by Thermo Fisher following manufacturer's protocol. Primers for full-length *Sr22* were designed with the help of Snap-Gene software. A fragment of 2823 kb was amplified using primers: *Sr22*-F 5′-ATGGCGGAAGTTCTGGTGAG and *Sr22*-R 5′-CTAGGACTTGACGGCCGGC.

Ligation in pUC57

pUC57 was used as a cloning vector and treated with *Eco*RV. *Sr22* gene eluted from gel was ligated into the cloning vector and after successful ligation pUC57-*Sr22* was transferred in *E. coli* for the confirmation of clones. *Sr22* genes from Mexi-Pak and Auqab 2000 were kept and examined separately.

Selection and Confirmation

Blue white colonies selection method was employed to identify pUC57-*Sr22*. The transgenic bacteria were placed on ampicillin-containing medium for screening. The confirmation of our desired clone was also achieved through restriction reaction. pUC57-*Sr22* was restricted with *Bam*H1 and *Nru*1 and run on agarose gel for confirmation through size.

Sequencing of pUC57-*Sr22*

pUC57-*Sr22* was isolated from bacteria and sequenced.

The sequence of *Sr22* from Mexi-Pak and Auqab 2000 were evaluated by finding similarity index using bioinformatics tools.

Results

Gene Frequency and Selection of Full-length Gene

The frequency of *Sr45* was highest among all other genes which was 65%. *Sr35*, *RPG1* and *Sr22* have gene frequencies of 56, 37 and 33% respectively, while *Sr33* and *RPG5* did not appear in any cultivar (Table 2). Among all studied genes, only *Sr22*, *Sr45*, *Sr33* and *Sr35* are likely to know as most stable and reliable genes for rust resistance. Screening of our germplasm showed that *Sr33* and *Sr35* were not present. *Sr22* and *Sr45* were present in some of the lines or commercial cultivars. *Sr45* is a much lengthier gene that is difficult to isolate, clone and transform. *Sr22* is comparatively smaller gene and therefore selected for further analysis.

Sr22 as Potential Gene for Transformation

Sr22 is highly potential and a race-specific resistant gene. *Sr22* is an inducible gene and active only when specific pathogen (*i.e*., *P. graminis*) attacks the plant. This gene is activated by some pathogenic proteins produced and induced in plant cells by *P. graminis* in order to prepare the host cells for defense. These proteins are called effectors. *Sr22* produces the protein of 106 kDa having 940 amino acids. The actual function of a *Sr22* protein is still unknown but one domain of this protein is similar to coiled-coil domain of the potato virus X resistance protein and similar proteins.

Isolation and Ligation of *Sr22*

cDNA was synthesized by H minus cDNA kit. *Sr22* was amplified by gene-specific primers using PCR and confirmed on gel by visualizing the band of 2823 bp (Fig. 1). Band of *Sr22* was eluted from the gel and ligated in pUC57 which was treated with *Eco*RV restriction endocuclease enzyme.

Cloning and Sequence Confirmation of *Sr22*

Transgenic *E. coli* cells were grown on selected medium containing ampicillin which allowed only those bacteria to grow who had transgenic plasmids. The sequence of *Sr22* was confirmed by sequencing the whole pUC57 that contained *Sr22*. The sequencing results confirmed the isolated fragment as the sequence of *Sr22*. The sequences form both Mexi-Pak and Auqab 2000 were almost identical except 3 bases in Auqab 2000 but protein sequence was identical in both cultivars. Both gene sequences were submitted in GenBank and allotted accession numbers (*i.e*., MH512000 and MH546067).

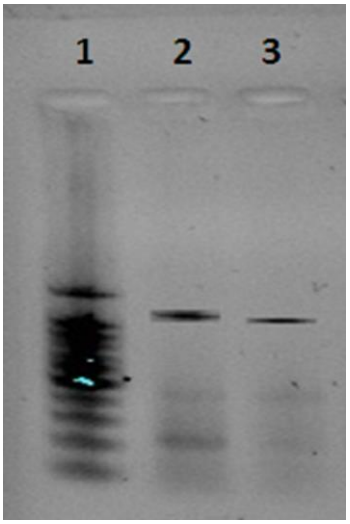

Fig. 1: cDNA amplification of *Sr22*: Lane 1: 100 bp DNA ladder (GenScript), Lanes 2 and 3: bands showing *Sr22* gene with approximately 2.823 kb size form Mexi-Pak and Auqab 2000 respectively

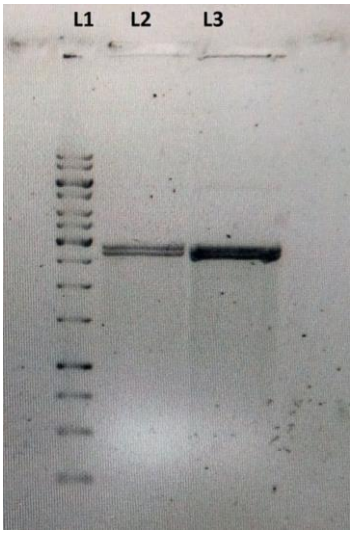

Fig. 2: pUC57-*Sr22* restricted with *Nru*1 and *Bam*H1 having 2 fragments: Lane 1: 100 bp DNA ladder (GenScript), Lane 2: gene of interest (*i.e*., *Sr22*) having size of 2842 bp, Lane 3: backbone of pUC57 having size of 2691 bp

Sequence Homology and Restriction Reaction of *Sr22*

The gene sequence of *Sr22* was compared with sequences present in GenBank database of NCBI using BLASTn program and some of its homologs have been shown in Table 3. Restriction enzymes *Nru*1 and *Bam*H1 were used for restriction reaction for pUC57-*Sr22* which resulted in two fragments. The sizes of both fragments were nearly close to each other (*i.e*., of 2842 bp and 2691 bp, respectively). The *Sr22* gene was found in the fragment of 2842 bp size (Fig. 2).

Table 1: List of primers used to amplify stem rust resistant genes in wheat

Primer name	Genes	Annealing Temp.	Primer Sequence (5′ – 3 ′)	Amplicon Size (bp)
Sr33-F	*Sr33*	56°C	GGGTTCCGCTGTATGGTAGA	373
Sr33-R			GATGATTGGGATGGGAGTTG	
Sr35-F	*Sr35*	56°C	AGGTTGTCGGGAAACTGTTG	374
Sr35-R			ATGGCCCAATTCTTTCACAG	
RPG1-F	*RPG1*	56°C	TCATGGATCCACCCTTTCAT	330
RPG1-R			TGCAACGATCACCATAAGGA	
RPG4-F	*RPG4*	56°C	CGTCCAGCTCCTTCTTGAAC	588
RPG4-R			AGGCACTCGTGCTCCTCTAC	
RPG5-F	*RPG5*	56°C	CTGCAGGTTCCTTTCCCATA	306
RPG5-R			GAGCAGAAAATCCTGCCTTG	
Sr22-F	*Sr22*	56°C	AGATCGGACCAGCCTGACTA	329
Sr22-R			AGGCAAGCGAAAAATAGCAA	
Sr45-F	*Sr45*	56°C	CCAAGATGCGGAGAAGAAAG	387
Sr45-R			TCTTCGTTTCTGGACCTGCT	

Table 2: The frequency of stem rust resistant genes in local wheat cultivars

Gene Name	Number of verities with presence of a particular gene	Number of verities with absence of a particular gene	%age of occurrence
Sr22	33	75	30.56
Sr33	0	108	0.00
Sr35	56	52	51.85
Sr45	65	43	60.19
RPG1	37	71	34.26
RPG4	18	90	16.67
RPG5	0	108	0.00

Table 3: Best matches of *Sr22* gene sequence given by BLASTn

Best matches	E-value	Identity (%)	Accession No.
Triticum aestivum mRNA, clone: tplb0012h21, cultivar Chinese Spring	0	96	AK456387.1
PREDICTED: *Aegilops tauschii* subsp. tauschii disease resistance protein RPM1-like (LOC109749436), mRNA	0	96	XM_020308399.1
Triticum aestivum cDNA, clone: WT012_L08, cultivar: Chinese Spring	0	95	AK335360.1
Hordeum vulgare subsp. Vulgare mRNA for predicted protein, complete cds, clone: NIASHv3150J07	0	92	AK377166.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_W3534	0	99	LN883755.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI330550	0	99	LN883752.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI190945	0	99	LN883749.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI289605	0	99	LN883751.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_IG44921	0	98	LN883748.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_IG44857	0	98	LN883746.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_IG44855	0	98	LN883745.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_SCHOMBURGK	0	98	LN883743.1
Triticum monococcum cultivar PI 306540 SrTm5 resistance protein (SrTm5) gene, complete cds	0	98	MG018615.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI573523	0	98	LN883754.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_IG44878	0	98	LN883747.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI355523	0	97	LN883753.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_DV92	0	97	LN883744.1
Hordeum vulgare subsp. Vulgare cDNA clone: FLbaf47f06, mRNA sequence	0	92	AK249642.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_PI272557	0	96	LN883750.1
Wheat stem rust resistance gene *Sr22* allele, isolate *SR22*_WESTONIA	0	95	LN883756.1

Discussion

Wheat production is greatly threatened by wheat stem rusts (Sr) which are dangerous pathogens and caused by fungus *P. graminis* f. spp. *Tritici* (*Pgt*). The fungal disease affects wheat production all over the world and causes loss in yield even up to 100% in susceptible varieties (Kimani *et al*., 2015). About 60 different *Sr* genes have been molecularly identified and studied so far in wheat. Several of these *Sr* genes provide wide spectrum resistance to plant at its all developmental stages such as *Sr26*, *Sr32*, *Sr39*, *Sr40* and *Sr47*. *Sr22* and *Sr45* (together with *Sr33*, *Sr35* and *Sr50*) are two of five major dominant Sr genes which have been cloned in wheat so far (Periyannan *et al*., 2013; Saintenac *et al*., 2013). All five genes exhibit resistance to Ug99 race group of *Pgt* while *Sr22*, *Sr33* and *Sr50* exhibit wide spectrum resistance to a number of pathogen races (Steuernagel *et al*., 2016).

The recurrence of wheat stem rust which is not only a major biotic threat to wheat production also poses a risk to the production of barley (Hatta *et al*., 2018). When wheat *Sr22*, *Sr33*, *Sr35* and *Sr45* genes were transferred to barley they were revealed to function and showed race-specific disease resistance against *Pgt*. This finding suggested that

downstream signaling pathway(s) of nucleotide-binding and leucine-rich repeat domains in wheat and barley has necessarily remained conserved since the divergence of both species 10–14 million years ago (Hatta *et al*., 2018).

The most effective and sustainable strategy to minimize yield losses in wheat production caused by stem rust disease is breeding for effective and strong field resistance. Therefore, in this study, seven genes *Sr22*, *Sr33*, *Sr36*, *Sr45*, *RPG1*, *RPG4* and *RPG5* were screened. The genes of *Sr* series were lost by wheat cultivars during evolution procedure (Gerechter-Amitai *et al.,* 1971). Finding *Sr22* and other genes in hexaploid wheat is a great work. It is also a novelty of this study that screening was purely done by amplifying DNA sequences. Mostly *Sr* genes were screened before using linked regions which may not be part of the actual gene. Therefore, it can be hypothesized that these linked regions could be present but not the gene itself (Ejaz *et al.*, 2012). Important resistant genes are found in the wild relatives of modern breed wheat which impart resistant to pest diseases. The method of breeding wheat for a resistant variety is cost-effective, reliable and environment-friendly. It is also proved to be the best method after practicing repeatedly (Goutam *et al.,* 2015). Periyannan *et al*. (2014) has described two sequence targeted sites (STS) markers CSIH81-BM and CSIH81-AG which are linked to *Sr22* in restriction fragment length polymorphism (RFLP) experiment. These two markers amplified 237 bp fragments. Ejaz *et al*. (2012) studied 117 Pakistani genotypes using these markers but did not find any positive results. In present study, primers were designed from the gene itself and PCR amplification ensured the presence of the gene. About 30.56% genotypes showed the presence of *Sr22*. The difference in results between our study and those reported by Ejaz *et al*. (2012) was due to target genotypes which were totally different in both studies. Only AS2002 was common in both studies but in this study this cultivar showed the presence of *Sr22*. This difference was due to the methodology adopted by Ejaz *et al*. (2012) as they amplified only linked regions.

Steuernagel *et al*. (2016) has confirmed the presence of the *Sr22* gene in Schomburgk, the hexaploid wheat line which carries *Sr22* from diploid parent wheat cultivars *Triticum boeoticum* accession G-213. *T. monococum* or diploid and hexaploid cultivars that have parentage from wild or diploid wheat cultivars are the only sources of the gene. *Sr45* was present in 60.19% of cultivars giving evidence of presence of this gene. Xwmc222 and Xcfa2158 linked markers were used to indicate the presence of *Sr45* (Yu *et al.,* 2010). *Sr35* was present in 53.7% cultivars. Babiker *et al*. (2009) also studied SSR markers for *Sr35* included Xgwm2, Xgwm5, Xgwm30, Xgwm32, Xgwm114, Xgwm133, Xgwm155, Xgwm162, Xgwm369, Xgwm391, Xgwm480, Xgwm497, Xgwm666, Xbarc25, Xbarc45, Xbarc179, Xbarc284, Xbarc314, Xcfd2, Xcfa2076 and Xcfa2193 which amplified different bands from 105 bp to 280 bp. *RPG1* gene is also not much common in local cultivars as it appeared only in 34.26% cultivars. Brueggeman *et al*. (2002) studied *RPG1* flanking markers ABG704 and ABG077 which were used to find out presence of the gene. *RPG4* appeared as a rare gene in 16.17% cultivars only. *RPG4* is barley origin gene present on chromosome number 5H. This gene was linked with SSR markers as described by Steffenson *et al*. (2009). SSR markers RB-SSR1 and R4-22 STS were used to detect the presence of a gene which gave PCR products of 304 bp and 922 bp respectively. *Sr33* and *RPG5* did not appear in any cultivar used in this study. Steffenson *et al*. (2009) used sequence tagged site (STS) markers Rpg5-STS and R13-STS for detection of *RPG5* gene by amplifying 627 bp and 1337 bp fragments respectively.

Conclusion

Results disclosed that Pakistani wheat cultivars had strong relationship with wild ones as 56% of cultivars showed the presence of *Sr22* gene. The nucleotide sequence identity of *Sr22* gene from hexaploid cultivars (*i.e*., Maxi-Pak and Auqab 2000) was found to be 99% with previously reported *Sr22* gene from *Triticum monococcum*. Sr22 gene isolated in this study can be used to upgrade a high yielding commercial cultivar susceptible to stem rust. The finding of this study also creates opportunities to transfer breeding process in Pakistan to a new scientific level using molecular genetics approaches.

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