

Molecular Characterization of Wheat Germplasm against Cereal Cyst Nematode Using Diagnostic *Cre3spf/r* and Microsatellite Markers

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Abstract

Wheat carries genes for controlling pathogenic response against Cereal Cyst Nematodes (CCN). *Cre3* is one of the CCN resistant genes transferred from *A. tauschii*, located on 2L of D genome. In the present study, twenty wheat accessions those are predominantly grown in temperate climatic zone of Pakistan, have been characterized by using a gene (G4) that is located at the *Cre3* locus member of NBS-LRR sequences (diagnostic marker *Cre3spf/r*) and closely linked microsatellites markers (*Xgwm 301* and *Xgwm 320*) for *Cre3* dominant resistance gene against CCN. Diagnostic marker *Cre3spf/r* has shown the presence of *Cre3* gene in all resistance carrying genotypes. *Xgwm 301* amplified the fragment of 170 bp for 6 homozygous dominant individual's viz., TD-1, SD-8006, Marvi-2000, Moomal-2002, Inqilab-91 and Bhattai and 210 bp for heterozygous individuals whereas *Xgwm 320* has generated 226 bp band in all resistance carrying genotypes.

Keywords: CCN; *Cre3*; Diagnostic Marker; Wheat; Microsatellite Marker; *Xgwm 320*

Introduction

Cereal cyst nematode (CCN) complex is a serious pathogen to cereal crops, present mostly in temperate countries. CCN experienced to be more challenging in areas where routinely cereal cropping occurs. Cereal cyst nematode is host specific which feed, develop and infect cereals and other grasses (particularly wild oat). The bio-assay for showing resistant lines in breeding program is not much authentic and needs lot of time. A resistance gene governing against cereal cyst nematode named *Cre1* refer to a single dominant gene was first detected in wheat land race [1]. Other resistance genes known were including *Cre2*, *Cre5* and *Cre6* from *Ae. ventricosa* [2-4], *Cre3* and *Cre4* in *Ae. tauschii* [5], *Cre R* from *Secale cereale* [6], *Cre F* from the wheat cultivar Festignay [7] and *Cre 7* (syn. *Cre Aet*) in *Ae. triuncialis* [8].

It has been demonstrated that RFLP marker csE20 was closely linked to the *Cre3* gene. Genomic replicas of csE20 carrying inserts were revealed to contain sequences 120 bp at 3' end of the addition with reading frame referring a leucine rich repeats (NBS-LRR), for nucleotide binding site and it has the distinctive character of disease resistance gene of plants [9]. The sequences of NBS-LRR that closely linked to the *Cre3* on chromosome 2DL comprised of a small sequence family with homologues spread in another place in the wheat genome [10-13].

DNA sequence with two, three, four or five nucleotide tandem repeats are known as microsatellites, as simple sequence repeats or as short tandem repeats. These markers seem to be hyper variable, it become ideal for genome mapping and population studies due to the co-dominance and reproducibility characters. For genetic diversity estimation, SSR molecular marker were exploited in comparison with RAPD markers and have shown more promising results than other markers [14]. Microsatellite markers avoid the limitation of pleiotro-

pic effect of genes. A huge number of microsatellites have been constructed [15,16] and supported as a very effective substitute because it deals in fast and particular assortment of the target gene [17], without any delay for phenotypic expression and can give trustworthy outcomes on the single plant basis [18].

It is reported that resistance genes against cereal cyst nematodes (CCN) either linked or co-segregating are *Cre1* [19,20], *Cre3* [5,9], *Cre6* [4]. D genome-specific microsatellite markers linked with the (*Cre3*) CCN resistant gene was also utilized for the validation of its resistance against more than 200 accessions of wheat [21].

Materials and Methods

Biological material

Germplasm

A total of 20 wheat genotypes (cultivars) viz., Sarsabz (1), Pak-81 (2), TD-1 (3), 8006 (4), Kiran-95 (5), Mehran-89 (6), Bakhtawar-92 (7), Imdad-2005 (8), Anmol-91 (9), 1200/19/1 (10), 8012 (11), MH-97 (12), Inqilab-91 (13), Mommal-2002 (14), Sassui (15), Abadgar-98 (16), 4047 (17), TJ-83 (18), Marvi-2000 (19) and Bhattai (20) were used in the study. These selected cultivars are predominantly grown in temperate climatic zone of Pakistan. The germplasm was obtained from Nuclear Institute of Agriculture (NIA), Tandojam, Sindh, Pakistan.

DNA analysis

Leaves of twelve-day-old wheat seedling were collected in the green house, frozen in liquid nitrogen then powdered with a mortar and pestle. DNA extraction was done according to the CTAB protocol published by [22]. Information of primer sequences is available at (<http://wheat.pw.usda.gov/index.shtml>) and primers were synthesized by Integrated DNA Technologies, Inc, USA. For primer set *Cre3spf/r*, amplification and cycling conditions were according to the protocol [4]. For microsatellites, PCR profile was designed after making the reaction material in the 12 μ L quantity, carrying 0.5 U Taq polymerase, PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 μ g/ μ L of primers and 100 μ g of template DNA. The PCR cycle, 94°C for 4 minutes (1 cycle), 94°C for 1 minute, 46°C for 1 minute, 72°C for 2 minutes (35 cycles) and 72°C for 10 minutes amplified clear bands with the lowest amount of background non-specific product. This PCR cycle was then used in further analysis of microsatellite markers. PCR products were separated on 1.5% agarose gel in 1x TAE, stained with ethidium bromide, visualized under UV light and photographed. For SSR, high-resolution metaphor agarose and agarose were dissolved to prepare (2.5%) gel in TBE buffer by heating for 5 - 10 minutes in microwave. Ethidium bromide in the amount of 4 μ L was added after a little cooling and the solution was swirled for a few seconds. Solution was then poured in gel casting tray with comb insertion for well formation. Samples were loaded after adding loading dye. DNA fragments were separated by supplying 35V for 4 hrs. 50 bp ladder was used as a standard for microsatellite markers. After electrophoresis, gel was observed on UV trans-illuminator at a wavelength of 320 nm.

Results

The aim of this study is to tag the wheat genotypes for their potential against CCN by utilizing microsatellite markers (*Xgwm 301* and *Xgwm 320*) and diagnostic marker (*Cre3spf/r*) of CCN resistance gene *Cre3*, present on chromosome 2DL. These genotypes have already been screened against *Heterodera avenae* pathotypes (Ha 41 and Ha 12) phenotypically [23].

Results from the CCN bioassays were quite similar with diagnostic and SSR markers for all the wheat genotypes tested. The genotypes were scored resistant after bioassays viz., TD-1, SD-8006, Marvi-2000, Moomal-2002, Inqilab-91 and Bhattai, which have shown *Cre3spf/r* diagnostic (Figure 1) as well as *Xgwm-301* SSR marker bands (Figure 2) whereas in susceptible genotypes viz., Pak-81, Mehran-89, Bakhtawar-92, Sassusi and Abadgar-98 negative results were obtained. Sarsabz, Kiran-95, Imdad -2005, 1200/19/1, SD-8012, Anmol-91, MH-97, SD-4047 and TJ-83 have shown different degree of resistance and susceptibility in bioassay experiments. Furthermore, at the

molecular level the diagnostic marker produced bands similar to the resistant genotypes fragment length but the SSR marker *Xgwm-301* generated bands of larger length. This confirms the presence of resistance gene in the positive tagged genotypes.

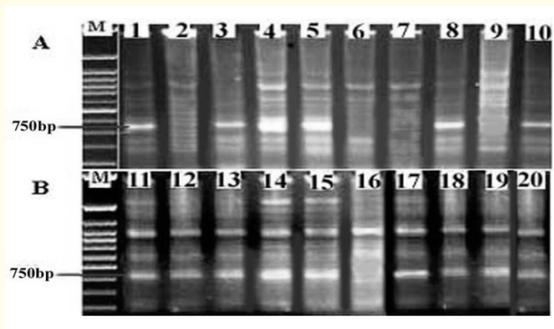


Figure 1: (A-B) Gel images showing banding pattern of resistant and susceptible wheat genotypes CCN Diagnostic marker; fragment length (750 bp for resistance gene).

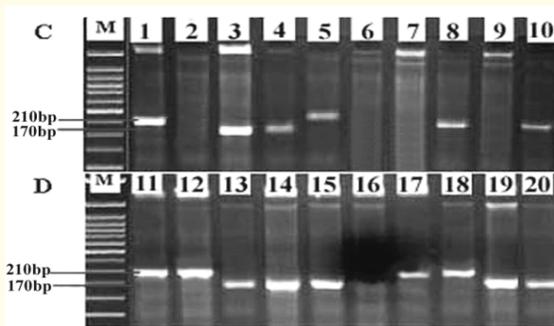


Figure 2: (C-D) Gel images showing banding pattern of resistant and susceptible wheat genotypes CCN Diagnostic marker; fragment length (210 bp and 170 bp for resistance gene).

The SSR marker *Xgwm-320* showed a different trend of banding-pattern for resistant and susceptible cultivars/lines. In resistant genotype (SD-8006), *Xgwm-320* fragment was absent. Similarly, Pak-81, Anmol-91 and Sassui were the susceptible genotypes with the presence of *Xgwm-320* fragment, while in other genotypes similar results were obtained corresponding to *Cre3spf/r* and *Xgwm-301* marker expression (Figure 3 and Table 1).

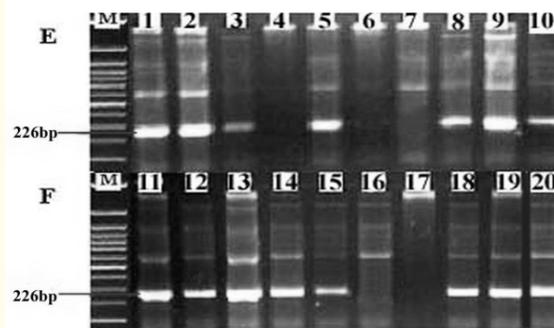


Figure 3: (E-F) Banding pattern of *Xgwm-320*; fragment length (226 bp for presence of allele).

Wheat genotypes	Bioassay rating*	Cre3spf/r	Xgwm 301 (bp)	Xgwm 320 (bp)
Sarsabz	Moderately resistant	+	210	226
Pak-81	Susceptible	-	-	226
TD-1	Resistant	+	170	226
SD-8006	Resistant	+	170	-
Kiran-95	Moderately resistant	+	210	226
Mehran-89	Susceptible	-	-	-
Bakhtawar-92	Susceptible	-	-	-
Imdad-2005	Moderately resistant	+	210	226
Anmol-91	Susceptible	-	-	226
1200/19/1	Moderately susceptible	+	210	226
SD-8012	Moderately resistant	+	210	226
MH-97	Moderately resistant	+	210	226
Inqilab-91	Resistant	+	170	226
Mommal-2002	Resistant	+	170	226
Sassui	Susceptible	-	-	226
Abadgar-98	Susceptible	-	-	-
SD-4047	Moderately susceptible	+	210	-
TJ-83	Moderately susceptible	+	210	226
Marvi-2000	Resistant	+	170	226
Bhittai	Resistant	+	170	226

Table 1: Association of microsatellite markers (*Xgwm 301* and *Xgwm 320*) with CCN resistance gene diagnostic marker (*Cre3spf/r*) in wheat genotypes.

* Erum and Shahina, 2016.

The primer pair *Xgwm-301* amplified an allele, approximately 170 bp fragment, for resistant genotypes and 210 bp for the genotypes with varied levels of resistance. *Xgwm-320* amplified 226 bp long fragment whereas the diagnostic marker generated the fragment of 760 bp for the *Cre3* gene. The results show that the link between *Xgwm-301* and the resistance gene was kept in all wheat genotypes that have potential against cereal cyst nematodes but it is not true for *Xgwm-320* SSR marker.

Discussion

The present study has described the application of diagnostic marker and SSR marker technique to screen indigenous wheat germplasm against CCN resistance. *Cre3* was chosen for this analysis as it is present on the D genome. Mc Fadden and Sears [24] proved that a large amount of analyzed data for resistance genes is present on *Aegilops tauschii* Coss., i.e. the donor of D genome of hexaploid wheat. The linkage of cs E20, as RFLP marker with *Cre3* resistance gene was reported on the distal arm of chromosome 2DL [5]. Cox [25] and Dvorak, et al. [26] made efforts to dissolve the issue of which biotype could be the source of high level resistance for different diseases. Pestsova, et al. [27], claimed that wild relatives (*Ae. tauschii*) of wheat represent a high potential source for crop improvement. Utilizing this all information, the efforts were extended to screen the targeted resistance gene against CCN present on 2DL i.e. *Cre3* gene and the

gene sequence marker *Cre3spf/r* was mapped by de Majnik, *et al* [28]. This resulted in an approach, focusing on the fixing of resistance gene in high yielding genotypes of indigenous germplasm. This information was supplemented to the present study and investigated the potential of resistance against CCN in indigenous wheat germplasm. Results obtained for the six genotypes (TD-1, SD-8006, Marvi-2000, Moomal-2002, Inqilab-91 and Bhattai) were resistant and almost consistent to the bioassay rating.

It is documented that majority of the microsatellite markers have proven to be inherited in a co-dominant manner [15,29]. Similarly, the SSR marker *Xgwm-301* was also inherited in co-dominant manner, because it is capable to detect homozygous as well as heterozygous resistance related bands. The microsatellite markers *Xgwm-301* used in the study has been proven efficient in identifying resistant genotypes whereas *Xgwm-320* was found to be less effective. In resistant genotypes, *Xgwm-301* has amplified 170 bp fragments whereas 210 bp bands were produced in genotypes with varying degree of resistance phenotypically.

This clears the close association of *Xgwm-301* with CCN resistance gene. The microsatellite marker, *Xgwm-301* amplified a fragment of 176bp is closely linked with the *Cre3* and on the linkage-map associated with the diagnostic marker that mapped within 4cM [30].

Xgwm-320 is another closest microsatellite marker to *Cre3* gene and mapped within 12cM away from the diagnostic marker on the linkage map [15,30]. Results from *Xgwm-320* differed from the bioassay and *Cre3* diagnostic marker in 4 out of 20 genotypes. SD-8006, the resistant genotype lacks *Xgwm-320* fragment whereas Pak-81, Anmol-91 and Sassui were the susceptible genotypes deficient in *Cre3spf/r* and *Xgwm-301* markers but showed *Xgwm-320* band. A possible explanation of distorted performance of this SSR marker is possibly due to the nucleotide sequence alterations within the priming recognition sites of susceptible plants. These alterations are due to the point mutation, insertions, deletions or inversions, which lead to no primer annealing and the absence of the corresponding band. As it was documented in the investigations of Martin, *et al.* [30] that *Xgwm-301* is closely linked with the *Cre3* gene mapped within 4cM and *Xgwm-320* mapped 12cM away from the diagnostic *Cre3spf/r* marker. It supports to prove that due to the high association of *Xgwm-301* with *Cre3* gene there is a lesser chance of crossing-over but since *Xgwm-320* is far away from the *Cre3* gene marker therefore, the chances of crossing over can exist in this case. In Pak-81, Anmol-91 and Sassui *Xgwm-320* inconsistent length fragment was seen to explain that the fragment was segregated in these genotypes.

It can be concluded that the screened genotypes against CCN using SSR markers (*Xgwm-301* and *Xgwm-320*) can be easily recommended to incorporate in the breeding schemes that will share in the overall selection gain and help plant breeders in saving time and expense. The presence of *Cre3* gene and *Xgwm-301* that conserved in indigenous germplasm shows that this marker can be efficiently applicable in control breeding strategies for the selection of CCN resistance. Therefore, it is imperative to tag the valuable genes using microsatellite markers that can help to develop varieties to meet the challenges of current and future agriculture.

Conclusion

Wheat germplasm comprised of 20 genotypes (cultivars and lines) were screened for genetic diversity against CCN by utilizing SSR marker technique. Six wheat genotypes viz., TD-1, Inqilab-91, SD-8006, Marvi-2000, Bhattai and Moomal-2002 are rated resistant due to the presence of diagnostic CCN marker *Cre3spf/r* and closely linked microsatellite markers (*Xgwm-301* and *Xgwm-320*) and recommended to incorporate essential data in biological yield potential program against CCN. Sound publicity of this potential will limit to reduce losses and effective for end users.

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