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Identification of molecular marker linked to stem rust resistance gene in synthetic hexapolid lines of wheat (*Triticum aestvium* L.)

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In order to enrich the molecular markers linked to wheat stem rust resistance gene and to facilitate the marker-assisted selection in breeding programme, bulk segregation analysis was carried out for gene tagging using F_2 segregating population derived from cross between wheat synthetic 4 line with resistant gene (*Sr* gene carrier) and a common wheat variety Agra local (susceptible to wheat stem rust). This *Sr* gene carrier synthetic 4 was derived between a cross *Triticum turgidum* (AABB) and *Triticum tauschii* (DD). A total of 35 SSR primers covering D and B Genome on the basis of chromosomal location of resistance genes were screened. The SSR marker namely *Xgwon106, Xgwm432, Xgwm533* and *Xgwm369* have revealed polymorphism among the parents used for study. The SSR *Xgwm 533* was found to be putative linked with stem rust resistance gene while the marker *Xgwm 389* was co- segregating with *Xgwm 533*. Marker *Xgwm* 533 and *Xgwm* 389 were taken from B genome. Hence, the stem rust resistance gene presumed to be located on 3-B genome of wheat. The use of these markers in combination with others markers could better predict the presence of stem rust resistance gene in wheat genome.

Key words: Stem rust (*Puccinia graminis* f.sp. *tritici)*, molecular tagging, bulk segregation analysis, synthetic hexaploid wheat, SSR primers.

INTRODUCTION

Wheat (*Triticum aestvium* L.) is one of the most important cereal food crop in the world and stem rust caused by *Pucinia graminis* sub sp. *tritici* has historically caused severe losses to wheat (*T. aestivum*) production worldwide. Thus, it posses a great threat to major proportion of wheat production globally. It has the potential to cause losses up to 100%, whereas leaf rusts up to 40% losses as reported by Singh et al. (2002). Stem rust has again become a major threat to global wheat production and food security with the emergence of the Ug99 lineage stem rust races that are virulent to most of the stem rust resistance genes deployed in wheat cultivars worldwide (Singh et al., 2011a; Xu et al., 2009; Botma et al., 2010). In India, stem rust occurs in most of the wheat growing areas, particularly severe in central, peninsular and southern part of the country. The introgression of resistance genes from related wild or cultivated species has provided wide genetic diversity for rust resistance in wheat. A single resistance gene present in a cultivar has always lead to emergence of new

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virulence races, it becomes necessary to identify, analyze and use the new resistance genes. Pyramiding of rust resistance genes through traditional phenotypic based technology is difficult when the different resistance genes produce similar infection type. This task can be assisted by identification of molecular marker linked to individual resistance genes through marker assisted selection (MAS) (Sharp et al., 2001; Babu et al., 2004). A cross of *T. turgidum* and *T. tauschii* was performed in order to diversify the genetic resistance and to exploit new genetic variability available for resistance or tolerance to abiotic and biotic stresses including rust. The D-genome of *T. tauschii* wheat and AB genome of *T. turgidum* are known to be a rich reservoir of valuable genes for resistance to diseases and pests of bread wheat (Cox et al., 1994). *Sr has* been transferred from the *Ae. tauschii* ssp. *tauschii* accession (Rouse et al., 2011; Eric et al., 2009), from *A. triuncialis* in wheat lines (Habibollah et al., 2012) and from Thatcher (Carmen et al., 2008) from Hordeum bulbosum (Fetch et al., 2009).

The present study was undertaken to identify molecular markers linked to the stem resistant gene in synthetic hexaploid line. Three Synthetic Hexaploid Wheat's (SHW's) viz. Synthetic 4, Synthetic 55, Synthetic 86 originated from CIMMYT, carrying high degree of resistance for stem rust were studied for their resistance against most virulent pathotype 40A. Among one resistance source Synthetic 4 was used to identify molecular marker linked to stem rust resistance gene.

MATERIALS AND METHODS

Plant material

Seeds of Synthetic 4, Synthetic 55, and Synthetic 86 were raised in sterilized medium in pots (6"x 6") containing decomposed agropeat, vermiculite and sand in the ratio of 2 : 1 : 1. Crosses were performed between Synthetic 4 (*Sr*-gene carrier) and Agra local (a susceptible variety) to raise the F_1 and F_2 population segregating for the stem rust resistance genes. The F_3 populations developed from the individual F_2 plants were maintained in the growth chamber under controlled conditions. The study was under taken during 2006 to 2009 at National Phytrotrone Facility, Indian Agricultural Research Institute, New Delhi.

DNA isolation

Freeze-dried leaf samples from 15 to 20 day old seedlings were ground into fine powder in liquid nitrogen and 50 mg of powdered tissue was used for isolation of total genomic DNA using the protocol described by Prabhu et al. (1998). The DNA was diluted to a final concentration of 10 ng/µl for SSR analysis.

PCR amplification and microsatellite marker analysis

Thirty five microsatellite markers covering distance 20 cM, present on the chromosome of D genome and B genome were selected. (Roder et al., 1998) were used to screen the parents. The PCR reaction was carried out in a 20 ml reaction volume containing 2.0 µl of 10X PCR buffer (10 µM Tris HCl, 50 µM KCL, 0.08% Nanidet P 40, 15 mM $MgCl_2$), 0.5-1.0 µl of 25 mM $MgCl_2$ (total 1.5 to 2.5 mM MgCl2 per reaction), 2.0 µl of each dNTP 0.3 µl of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 5 µl of template DNA (10ng/µl stock) and 40 ng of each SSR primers (10 ng/µl stock). Amplification was carried out in a PTC-200 thermal cycler programmed at 1 cycle of 3 min at 94°C, 55-60°C (depending on primer) for 1 min. And 72°C for 1 min (45 cycles) and a final extension step of 72°C for 10 min (1 cycle). PCR products were resolved on 3% Metaphore agarose (Cambrex Bio Science Rockland, Inc., USA) gel at 80v for 3 h. Gels were stained in ethidium bromide and photographed on a digital gel documentation system (Alpha Innotech, San Leandro, CA, USA).

Bulk segregation analysis

Bulk segregation analysis, as suggested by Michelmore et al. (1991), was employed to identify SSR marker linked to stem rust resistance gene in synthetic hexaploid lines of wheat. The homozygous resistant and homozygous susceptible F_2 plants identified based on F_3 progeny testing and were used for bulk segregation analysis. The resistant and susceptible bulks were constituted and tested with SSR primers along with their parents to identify putative SSR markers. The F_2 population from the cross Synthetic 4 x Agra local was subjected for a chi-square test for goodness of fit.

Statistical analysis

The Chi-square (χ^2) test for goodness of fit for a ratio (Panse and Sukhatme, 1967), was used to compare the actual ratios with those calculated for Mendelian segregation.The seedlings that were scored as resistant and susceptible were subjected to Chi-square (χ^2) test for goodness of fit to test the deviation of the observed segregation data from the theoretically expected segregation ratio.

Back ground information

Wheat line Synthetic 4 which was derived from a cross of *Triticum turgidum* (AABB) and *Triticum tauschii* (DD). The *Triticum turgidum* carries stem rust resistance gene *Sr2, Sr13* and *Sr14* while *T. tauschii* carries stem rust resistance gene *Sr33* (IDS) and *Sr 45* (Simons et al., 2011).

RESULTS AND DISCUSSION

Parental polymorphism

The parental polymorphisms among three parental lines namely; Synthetic 4, Synthetic 55, Synthetic 86 along with Agra local were studied and a total of 75 RAPD's and 35 Micro-satellite covering both D and B genome of wheat were screened. The RAPD markers selected have not produced any reproducible result due to lack of reproducibility as a result of the microsatellite markers were under taken for this analysis. Microsatellite markers/SSR markers show co-dominant expression and multiallelism, highly polymorphic, genome specific, abundantly distributed throughout the genome and have recently become important genetic markers in cereals

Table 1. The SSR markers with their genome location showing polymorphic among the parental lines and Agra local.

S/N	SSR markers	Primer sequence (5'……3')	Amplification product size (bp)	AT (°C)
1	XGMW 106-1D	F'5'CTG TTC TTG CGT GGC ATTA AA 3' R5'AAT AAG GAC ACA ATT GGG ATG G 3'	60	55
2	XGWM 533-3B	F5' AAG GCG AAT CAA ACG GAA TA 3' R5' GTT GCT TTA GGG GAA AAG CC 3'	120	60
3	XGWM 389-3B	F5' ATC ATG ATC TCC TTG ACG 3' R5' TGC CAT GCA CAT TAG CAG AT 3'	117	60

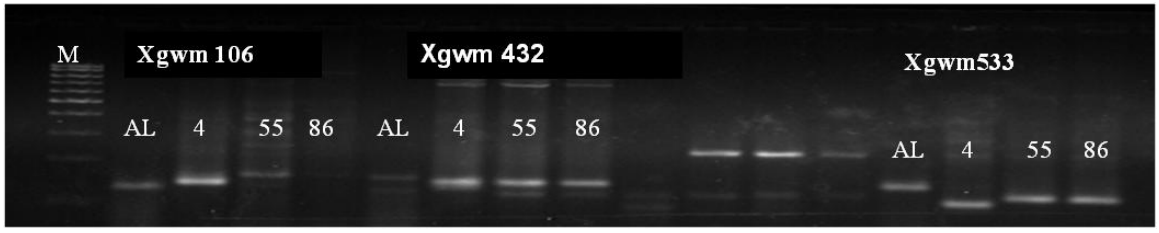

Figure 1. The Microsattelite markers showing polymorphic among the parental lines; Agra local, Synthetic 4, Synthetic 55 and Synthetic 86. M- 100 bp leader; AL- Agra local; 4- Synthetic 4; 55- Synthetic 55; 86- Synthetic 86 and SSR markers × Xgwm 106; Xgwm 432 and Xgwm 533.

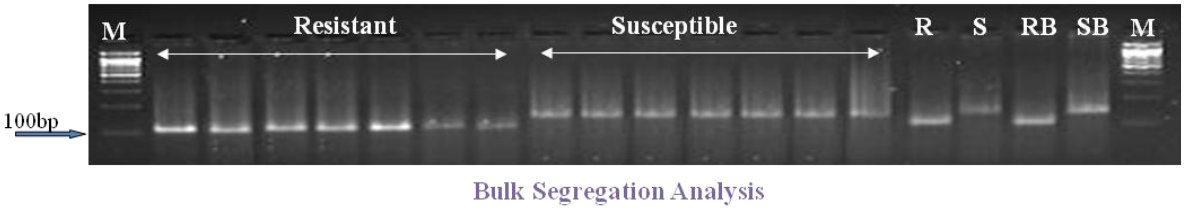

Figure 2. The SSR marker *Xgwm 533* linked to the stem rust resistance gene in resistant bulk (RB) and susceptible bulk (SB), Synthetic 4 and Agra local. M- 100 bp leader; AL- Agra local; 4- Synthetic 4; 55- Synthetic 55; 86- Synthetic 86 and SSR markers × Xgwm 106; Xgwm 432 and Xgwm 533.

breeding including wheat and because of these characteristics, simple sequence repeats markers/microsatellite markers exhibit high PIC values. These markers display high gene diversity scores which make them useful in distinguishing closely related genotypes. SSR marker namely *Xgwon106, Xgwm432, Xgwm533 and Xgwm369* have exhibited good polymorphism among the parental lines used for study (that is, Synthetic 4, Synthetic 55, Synthetic 86 and Agra local) as indicated in Table 1 and Figure 1.

Gene tagging using bulk segregation analysis

In the bulk segregation analysis (BSA), resistant and susceptible bulks were constituted using homozygous resistant and homozygous susceptible plants. Twenty three individuals were homozygous for the resistance allele identify by progeny testing in F_3 generation and therefore taken to constitute resistant bulk. Similarly, twenty three individuals homozygous for susceptible alleles were taken constitute susceptible bulk used for tagging of the resistance gene. A total of 35 SSR primer selected at a regular interval from D and B genome of wheat (Roder et al., 1998), only primers *Xgwm533* has reveled the detectable polymorphism among both the resistant and susceptible bulks as well as in parental lines (Agra local and Synthetic 4) (Figure 2). Due to marker *Xgwm 389* was co segregating with *Xgwm533.* Which indicates these two markers; *Xgwm 533* and *Xgwm 389* were putative linked for stem rust resistant gene in Synthetic line 4. These results were in conformity with Dhillon and Dhaliwal (2011) for "Identifying the AFLP Markers Linked to Leaf Rust Resistance genes using

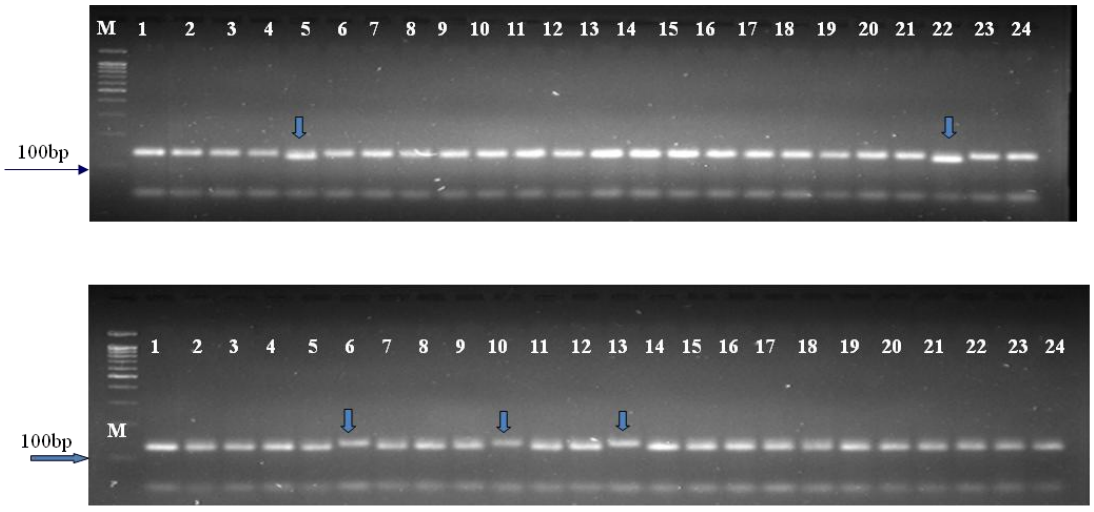

Figure 3. Flanking marker *Xgwm 389*, showing segregation in F_2 susceptible plants from left to right. M- 100bp leader; Arrow- indicate recombinant types.

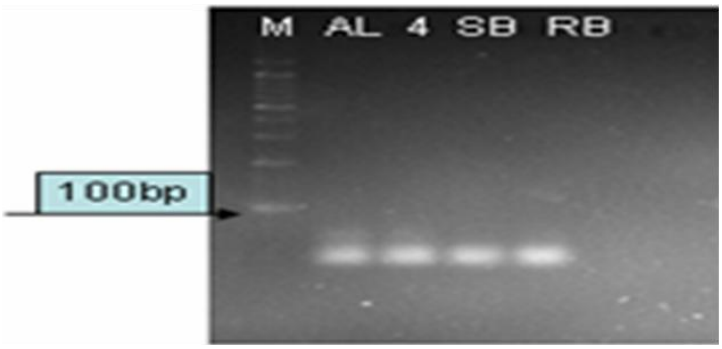

Figure 4. The absence of stem rust resistance gene *Sr* 33 gene conferred by STS primer *Xabc 1560 1DS.* M- 100 bp leader; AL- Agra local; 4- Synthetic 4; SB- Susceptible bulk, RB- Resistant bulk.

near Isogenic Lines of wheat. Similarly, Rouse et al. (2012). Identify the markers linked to the race Ug99 effective stem rust resistance gene *Sr28* in wheat (*Triticum aestivum* L.) and Leonardo et al. (2011) have Identify the leaf rust resistance genes in selected Argentinean bread wheat cultivars by molecular markers.

The *Xgwm533* primer was also screen for its segregation in the entire set of selected F_2 individual seedlings. The data shows presence of the critical bands in 44 individual out of the 48 resistant F_2 plants and 4 individuals reported with susceptible type. Similarly, the presence of susceptible band in 44 individual of the 48 susceptible F_2 plants and four individuals with resistant type, indicates presence of a total eight recombinant. The result indicates marker *Xgwm533* is located at a distance of 8 cM from the resistance gene. The adjacent marker *Xgwm389* (located on 3B genome) was also screened for its segregation patterns in F_2. The data showed the presence of the existing bands in 42 out of the 48 resistant F_2 plants, which were equal to the resistant band. While the presence of susceptible band in 44 out of 48 susceptible F_2 plants indicate presence of a total ten recombinant (Figure 3). It indicates that marker *Xgwm389* is located approximately at the distance of 10 cM or more from the stem rust resistance gene.

Based on bulk segregation analysis, it could be predicted that stem rust resistance gene in synthetic 4 is located on 3B genome of wheat. Since these two markers that is, *Xgwm533* and *Xgwm389* are located on 3B genome of wheat. The possibility of *Sr2* could be ruled out as *Sr2* carrying plant shows pseudo-black chaff (PBC) a dark pigmentation developed around stem internodes and glumes was not reported during seedling stage. Therefore, it might be presumed that it could be a new gene other than *Sr2* which come from 3B genome of wheat. The possibility of presence of *Sr* 33 was ruled out by using STS primer *Xabc* 156-1D which is tightly linked to *Sr* 33 gene (Figure 4). Using molecular linked with stem

rust resistance genes, it is much easier to trace the resistance gene in segregating population. Thus, the use of these markers in combination with other could predict the presence of gene for stem rust resistance in breeding population. Further mapping attempts in this chromosomal region with more markers and larger F_2 and/or $F_{2:3}$ sample sizes are warranted to identify closer and more efficient markers for breeding population.

Conclusion

It is essential to have a molecular marker for these resistant genes for the sake of convenience in introgression or in gene pyramiding to identify a SSR marker for the resistant gene in F_2 mapping population. In bulk segregation analysis, SSR marker *Xgwm 533* was found to be putative linked with stem rust resistance gene while the marker *Xgwm 389* was co-segregating with it. These markers were taken from B genome. Hence, the stem rust resistance gene in Synthetic 4 presumed to be come from 3-B genome of *Triticum turgidum*- (AABB).

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