

MOLECULAR IMPLICATIONS FROM SSR MARKERS FOR STRIPE RUST (*PUCCINIA STRIIFORMIS* F.SP.*TRITICI*) RESISTANCE GENE IN BREAD WHEAT LINE N95175

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Abstract

Stripe rust caused by *Puccinia striiformis* f. sp. *tritici* is one of the most devastating diseases of wheat in China as well as in Pakistan. In the present studies F₂ population was established by crossing N95175 resistant to stripe rust race CYR32 with two susceptible lines Huixianhong and Abbondanza to molecularly tag resistance gene existing in wheat line N95175. The segregation of phenotype was accorded with an expected 3:1 ratio in both combinations studied and fit the model of a single dominant gene controlling stripe rust resistance in N95175. Thirty five SSR primer pairs were screened on the parents and bulks and also on individuals since resistance gene to be located in chromosome 1B. The result indicated that most of resistant plants amplified same band as resistant parent while susceptible plants amplified same as susceptible parents studied and considered that markers co-segregated with resistant loci in N95175. This yellow rust resistance gene was considered to be Yr26 originally thought to be also located in chromosome arm 1BS linked to marker loci Xgwm273 and Xgwm11 with genetic distances ranging from 1.075cM to 2.74cM in both combinations studied. However, the closest loci were observed 2.67cM for Xgwm273 and 1.075cM for Xgwm11 in Huixianhong XN95175 and Abbondanza XN95175 crosses respectively. Hence, it has been concluded that the PCR-based micro satellite markers Xgwm273 and Xgwm11 located in chromosome 1B were shown to be very effective for the detection of Yr26 gene in segregating population and can be applied in future wheat breeding strategies.

Introduction

Wheat crop is grown on more than 40% of the cultivated area (8.307 million hectare) of Pakistan with an average production of 2,615 kg/hectare (Jamali *et al.*, 2007). Although a bumper crop was harvested in 2006-07(23.5 million tons) but the average production is quite low in comparison with other agricultural countries due to lot of biotic and a-biotic stresses. Thus production and supply of transgenic plants resistant to biotic and a-biotic stresses is an essential element for sustainable production. Increased production will significantly contribute to food supply for the rapidly increasing population as well as in the foreign exchange earning for the country.

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is one of the major limitations for wheat production throughout the world. The application of resistance genes in wheat breeding is the most effective, economical and environmentally friendly approach for controlling this disease (Chen *et al.*, 2005). In China, especially in southwest China, stripe rust is the most commonly occurring disease due to temperate environmental condition in wheat growing season. Yield loss of wheat can be as high as 20 to 30% in the years when stripe rust occurs only moderately epidemic on susceptible cultivars. Therefore, introducing new sources of resistance to stripe rust is essential for durable

resistance (Chunmei *et al.*, 2008; Jianxin *et al.*, 2001 and Peng *et al.*, 1999). To date more than forty three resistant genes at different loci have been designated and mapped to different wheat chromosomes, out of which most of genes are race- specific and cultivars possessing some of them played important role in wheat breeding (Cao *et al.*, 2001; McIntosh *et al.*, 1996; 2003; 2004; 2005). However, most of the race specific genes have become ineffective in a period of time when extensively used in production due to appearance of different virulent races (Wu & Niu 2000; Chen *et al.*, 2001; Wang *et al.*, 1996; Niu & Wu 1997 and Daolin *et al.*, 2009).

The discovery of DNA as hereditary material followed by its sequence organization on chromosomes opened new avenues in the field of molecular genetics. During last two decades, enormous information have been generated and incorporated to strengthen many research areas in the field of agriculture in general and plant breeding in particular. One of the most effective tools is the integration of marker assisted selection (MAS) which laid down the foundation of plant molecular breeding. Molecular markers not only allowed the easy and reliable identification of clones and breeding lines but also facilitated the monitoring of introgression and the estimation of genetic diversity and relatedness among germplasm (Mukhtar *et al.*, 2002).

Simple sequence repeats (SSR), or micro satellites, are useful tools for molecular genetic analysis, as they are more abundant and display higher levels of polymorphisms in many plant species (Hitta *et al.*, 1995; Chen *et al.* 1999; Kam-Morgan *et al.*, 1989 and Plaschke *et al.*, 1995). SSR markers have been reported for several stripe rust resistance genes, including *Yr5*, *Yr10*, *Yr15*, *Yr24* and *YrH52* (Sun *et al.*, 2002; Wang *et al.* 2002; Peng *et al.*, 2000, 1999; Zakari *et al.*, 2003), out of which some markers have been used in marker-assisted selection and for pyramiding resistance genes, as well as for understanding of the relationships among different genes. Molecular markers linked many stripe rust resistance genes have been reported by different authors available online from the U.S. Department of Agriculture. In hexaploid wheat, simple sequence repeats are more informative and useful than any other marker system in molecular mapping because of their high polymorphism. The purpose of present study was to identify novel genes resistant to stripe rust in wheat line N95175 by using micro-satellite markers.

Materials and Methods

Plant material: Wheat variety/line N95175 carrying resistance gene against stripe rust produced from the cross 92R149/Xian87 (130)// Xiaoyan No. 6 and seed of F₂ population derived from the crosses with Huixianhong and Abbondanza produced and provided by the College of Agronomy, Northwest A&F University, Yangling Shaanxi, China was used. The 215 F₂ plants of two crosses were used to construct population for bulk segregating analysis.

Testing for resistance: The randomly selected seeds of all above plants were sown in small pots under controlled conditions isolated by glass enclosure (3.6 fits in height) in the greenhouse at the Plant Protection College, Northwest A&F University Yangling, Shaanxi, China. Seedlings were inoculated at the three-leaf stage, using an equivalent mixture of urediospores of the Yellow stripe rust race CYR32 mixed with talcum powder at 1:1 ratio and incubated at 9°C and 100 / relative humidity (RH) for 24 h and then transformed into a green house maintained with 14h light (22,000lx) at 17°C and 10h of darkness at 12°C with 70/ RH.

When the pustules of stripe rust were fully developed and easily discerned at the seedling stage the infection types (Its) were recorded 14-15 days after inoculation according to the standard classification system from 0, 0; 1; 2; 3; and 4 Infection type scale. IT 0 represented no visible symptoms; 0;, necrotic flecks; 1, small sporulating uredia surrounded by necrotic tissue; 2, small to medium uredia with chlorosis and necrosis; 3, moderately sized sporulating uredia surrounded only by chlorotic tissue; and 4, abundantly sporulating uredia without chlorosis. Hence 0-2 indicating resistance and 3-4 indicating susceptible followed by Bariana & McIntosh (1993).

Preparation of genomic DNA samples: DNA samples were extracted from green leaves of three parents (N95175, Huixianhong and Abbondanza) and their respective resistant and susceptible F₂ individuals as described by Sharp *et al.*, (1988). Resistant and susceptible bulks for bulk segregate analysis (BSA) were made by pooling equal amounts of DNA from 10 resistant and 10 susceptible plants (Michelmore *et al.*, 1991) respectively from the segregating F₂ population and analyzed in comparison with individuals and respective parents for further investigation.

PCR amplification and electrophoresis separation of products: Thirty five primer pairs of SSR located on chromosome 1B in wheat variety N95175 were screened on the parents and bulks and also on the individuals since *Yr26* was known to be located on chromosome 1B (Ma *et al.*, 2001). The SSR analysis followed the procedure of Bryan *et al.*, (1997) with some modifications. PCR reactions were performed in 10 μ L volume in a Perkin Elmer 480Thermocycler. The reaction solution contained 10mM/LTrisHCl, 50mM/LKCl, 2Mm/L MgCl₂, 200 μ mol/L of each de-oxy-nucleotide, 250nM/L of each primer, 20 to 40ng genomic DNA, and 0.25U *Taq* DNA polymerase. The amplification were performed at 95°C for 3 min, firstly followed by 5 cycles at 94°C for 1 min, at 47°C to 60°C (depending on the micro satellite primers) for 1.5 min., and at 72°C for 1 min., secondly followed by 30 cycles at 92°C for 30s, at 47°C to 60°C (depending on the micro satellite primers) for 50s, and at 72°C for 30s, with a final extension at 72°C for 5 min. PCR products were mixed with one fifth volume of loading buffer (100mM/L EDTA pH 8.0, 10mM/L TrisHCl pH 7.5, 5% Ficoll 400; 0.05% bromophenol, 0.05% xylene cyanol) and 10 μ L were loaded for electrophoresis in vertical, no denaturing 8% polyacrylamide gels in 1 \times TBE (90mM/LTris borate pH 8.3, 2mM/L EDTA) at 50mA for 2 to 3 h (Wang *et al.*, 2007).

Silver staining and linkage analysis: PCR products were analyzed by Poly-Acryl-amide Gel Electrophoresis (PAGE). Band patterns were visualized with silver staining. Briefly, the gel on a glass plate was pretreated with fix/stop solution [10% alcohol and 0.5% acetic acid (v/v)] for 10 min., and then stained in the 0.2% AgNO₃ Solution for 15 min. After a brief rinse in distilled H₂O for 1 min, the gel was transferred into a solution containing 0.002% (w/v) of sodium thiosulfate for 1 min followed by incubation in the well chilled developer solution (15%(w/v) sodium hydrate and 0.4% (v/v) Formaldehyde) for 3-8 min. The reaction was stopped by incubating the gel in distilled water by shaking for 5 min.

Chi squared (X^2) tests were applied to the inheritance data to establish goodness of fit to postulate ratios (Bariana *et al.*, 2006). Genetic distances were calculated by means of the Kosambi mapping function.

Results

Resistance response of the analyzed wheat lines and cultivars: The wheat cultivars N95175 was highly resistant (IT.0), whereas Huixianhong and Abbondanza were susceptible (IT.3-4) to stripe rust race CYR32, at seedling stage. In the 215 tested F₂ plants, the cross Huixianhong X N95175 produced 93 individuals highly resistant and 25 individuals were susceptible, whereas in cross Abbondanza X N95175, 75 individuals were highly resistant and 24 were highly susceptible to stripe rust race CYR32, at seedling stage. The ratios of resistant and susceptible F₂ plants of the two crosses were 93:25 and 75: 24 respectively. The segregation of phenotype was accorded with an expected 3:1 ratio in both combinations studied and fit the model of a single dominant gene with values of $\chi^2=0.915$ and $P=0.339$ for Huixianhong X N95175 F₂ generation whereas $\chi^2=0.278$ and $P=0.598$ for Abbondanza X N95175 F₂ generation studied.

SSR analysis: In the present studies, 35 micro satellite primer pairs were screened, out of which two primer pairs *Xgwm273* and *Xgwm11* showed polymorphism between resistant and susceptible plants. However, these primers were used to evaluate polymorphism between susceptible and resistant bulk pools as well as their parents and individuals respectively and showed clear polymorphisms, hence entire F₂ population was then genotyped with the polymorphic markers. Most of the susceptible plants amplified the same bands as the susceptible parents, similarly resistant plants amplified same bands as the resistant parent (homozygous) or both (heterozygous) hence obviously cleared polymorphisms have been observed among the all resistant and susceptible genotypes studied (Fig. 1). The result indicated that both the markers loci were co-segregated with the resistance gene in wheat line N95175.

The SSR marker linkage analysis indicated that the temporary designed resistance gene *Yr26* was closely linked to markers *Xgwm273* and *Xgwm11* loci with genetic distances ranging from 1.075 to 2.74cM in both the combinations studied (Table 1). The closest loci were observed as *Xgwm273* with 2.67cM and *Xgwm11* with 1.075cM in the crosses Huixianhong X N95175 and Abbondanza X N95175 respectively. Consequently, the resistant gene was assumed to be in chromosome 1BS (Ma *et al.*, 2001) because the flanking marker loci were in chromosome 1BS (Fig. 1). Hence, it has been concluded that these PCR-based micro satellite markers *Xgwm273* and *Xgwm11* located in chromosome 1B were shown to be very effective for the detection of the *Yr26* gene in segregating populations and can be applied in future wheat breeding strategies.

Table 1. F₂ plant segregation for stripe rust resistance gene and polymorphic marker.

Phenotypes	Huixianhong X N95175				Abbondanza X N95175			
	A-B,	A-bb,	aa-B,	aabb	A-B,	A-bb,	aa-B,	aabb
Xgwm273	92	3	0	25	75	2	0	20
Xgwm11	92	2	1	23	74	1	0	22

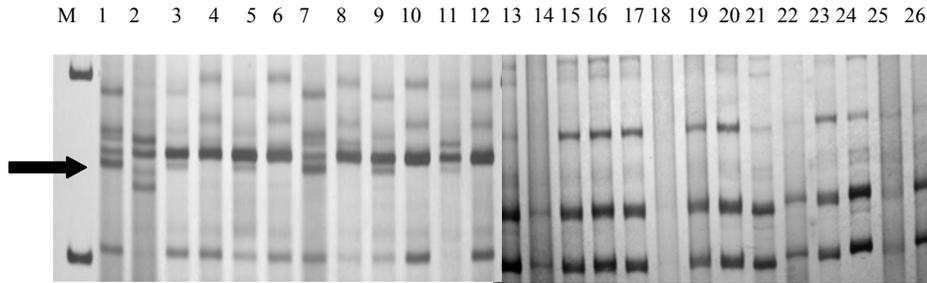


Fig. 1. The result analysis of micro satellite loci Xgwm273 and Xgwm11: The lanes. 1,7,13 and 21 for parent N95175, while 2, 14, 8 and 22 for parents Huixianhong and Abbondanza. Hence, 3, 15, 9 and 23 resistant bulks while 4, 16, 10 and 24 for susceptible bulks of both the crosses for both markers respectively. Hence, lanes 5,17,19,11, and 25 for Resistant, while 6,18,20,12 and 26 for susceptible F_2 plants in both the crosses for both markers respectively. The 2000bp marker was used.

Discussion

A number of stripe rust resistance genes have been identified and incorporated into different commercial cultivars. To date, more than forty loci for stripe rust resistance have been published officially and several other genes have been named provisionally available online from the U.S. Department of Agriculture (Chunmei *et al.*, 2008). In the present studies, we used F_2 population with micro satellite markers to molecularly tag the temporarily designated stripe-rust resistance gene *Yr26* (Ma *et al.*, 2001) existing in wheat line N95175. The wheat line N95175 displayed highly resistance whereas the lines Huixianhong and Abbondanza were highly susceptible against stripe rust race CYR32.

According to pedigree of the cultivar N95175 which was derived from a local variety 92R149 reported as resistant to many stripe rust races like CYR30, CYR31 and H46-4 (Luo, 2005) proved highly resistance against stripe rust race CYR32 also, whereas, rest of the lines taken under consideration were not carrying such type of genes hence exhibited high susceptibility against stripe rust race studied. Moreover, the temporarily designated stripe-rust resistance gene *Yr26* resistant to the stripe rust race CYR32 in wheat line N95175 was mapped by using 35 micro-satellite markers. Two primer pairs Xgwm273 and Xgwm11 out of 35 tested SSR primer pairs showed polymorphism and co-segregated with resistant loci in both combinations studied. Then the entire F_2 population was genotyped with these two Xgwm273 and Xgwm11 polymorphic markers and observed that most of susceptible plants amplified the same bands as the susceptible parents while most of the resistant plants amplified same bands as the resistant parent N95175, which may be because of no cross over between resistance gene and polymorphic marker loci detected in this study. Hence, it has been concluded that the resistance gene in N95175 was closely linked with micro satellite markers Xgwm273 and Xgwm11 located on chromosome 1B (Le *et al.*, 2006, Ma *et al.*, 2001 and Zakari *et al.*, 2003). Moreover, the results also indicated that the Xgwm273 and Xgwm11 were diagnostic markers for resistance to stripe rust race CYR32 in N95175, hence can be utilized as linked markers in future marker -assisted selection strategies to encounter the stripe rust race CYR32 in wheat.

Ma *et al.*, (2001) reported that *Yr26* was located in 1BS based on linkage with SSR marker loci *Xgwm11*, *Xgwm18* and *Xgwm413* with genetic distances of 1.9 cM, 1.9 cM and 4.3 cM, respectively, in a 110 plant F₂ population of Yumai 18 X R55 (92R137) inoculated with stripe rust race CYR29. It has been reported by other workers that the resistance gene *Yr26*, *Yr24* and *YrCH42* were likely to be identical because lines with them gave similar reaction patterns with *Yr26* located on chromosome 1B (Le *et al.*, 2006, Ma *et al.*, 2001 and Zakari *et al.*, 2003). Work in Australia also indicated that *Yr26* and *Yr24* were also identical (McIntosh *et al.*, pers.com). Keeping in view of importance of these findings, it can be assumed that *Xgwm273* and *Xgwm11* are linked to *Yr26* because both the flanking markers were existing at chromosome arm 1BS. These results were not according to the results reported by Chunmei (2008), in which the order for *Yr26* was reversed and linked marker loci *Xgwm11* - *Xbarc187* - *YrCH42* - *Xgwm498* - *Xbarc240*, were located on chromosome 1BL. A possible reason for the discrepancy in gene order could be because of different pattern of gene flow and the rate of cross over in different genetic sources.

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