Identification of quantitatve trait loci associated with leaf rust resistance in wheat (*Triticum aestivum L*) using microsatellite markers

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ABSTRACT

This study is conducted to identify quantitative trait loci (QTLs) for leaf rust resistance in F2 population of wheat by using bulked segregant analysis (BSA) with simple sequence repeat (SSR) markers. F2 population were resulted from a cross between the variant line (S11), which is susceptible to leaf rust and the resistant (CIMMYT) variety Pavon 76. F2 of 101 individuals along with their parental lines were analyzed with thirty three pairs of simple sequence repeats (SSRs) markers. The results showed that only two of SSR primer pairs (Xgwm 428 and Xgwm334) gave polymorphic DNA fragments. A simple single marker regression analysis showed that these two markers were found to be linked to leaf rust QTL (LrD and Lr A). R^2 explained by markers Xgwm 428 and Xgwm 334 were 43 and 55%, due to the segregation of the QTL respectively. The LrD and LrA genes, mapped on chromosome 7D and 6A respectively, may act in a complementary interaction.
Keywords: Leaf rust resistance – QTL - SSR markers- *Triticum aestivum*.

INTRODUCTION

Leaf rust, caused by *Puccinia triticina* Eriks is one of the most common foliar diseases of bread wheat (*Triticum aestivum*) in the world (Mebrate *et al.*, 2008). Breeding for resistance is considered to be the most economical and environment- friendly strategy for disease control.To date, more than sixty leaf rust resistance genes have been identified in wheat (McIntosh *et al.*, 2008; Samsampou *et al.*, 2010) . Many of these resistance genes are effective at both the seedling and adult plant growth stages and exhibit hypersensitive reactions that facilitate the emergence of virulent pathogen mutants, which can rapidly overcome the resistance (Lagudah, 2011). For that reason, to obtain wheat cultivars with effective resistance for a long period of time, novel genes from different resistant sources can be identified, introgressed to these cultivars, and the identification of gene combination which can lead to increase the level of resistance (Messmer *et al.*, 2000; McIntosh *et al.*, 1995; Kolmer and liu,2002; Oelke and Kolmer, 2005). A few genes confer resistance during the adult plant growth stage and have the capacity to express slow-rusting resistance (Singh *et al.*, 2011). In most cases, if the gene is recessive or partially recessive, it exhibits continuous variation in segregating populations and is under oligogenic control (Bjarko *et al.*, 1988). To date, 4 slow-rusting adult plant resistance (APR) loci (resistant to leaf rust) have been identified, given gene designations, and mapped to specific genomic locations. These 4 loci are *Lr34*, *Lr46*, *Lr67*, and *Lr68* (William *et al.*, 2006; Krattinger *et al.*, 2009; Herrera-Foessel *et al.*, 2011, 2012).

Simple sequence repeats (SSRs) distinguished by high level of polymorphism and high interspersion rate, this make them an abundant source of genetic markers. Microsatellites provided more polymorphism than RFLP or RAPDs, probably because of the complex nature of the wheat genome (Imtiaz *et al.,* 2001). Many of leaf rust resistance genes were mapped by using SSR such as Lr39, Lr 34, and Lr 63 (Raupp *et al.*, 2001; Suenaga *et al.*, 2003, and Kolmer *et al.*, 2010).

Quantitative trait locus (QTL) analysis is a powerful tools to identify genomic regions and chromosomal locations such as Lr 34 and Lr 46 with major effect mapped on 7DS and 1BL respectively. Additional minor QTLs were identified (Suenaga *et al.*, 2003; Schnurbuch *et al.,* 2004). Bulked segregant analysis (BSA) is a method to identify molecular markers linked to a gene of interest without having to construct a map of the genome (Michlemore *et al.*, 1991). BSA and linkage mapping in wheat has enabled identification of molecular markers linked to genes that condition resistance to leaf rust (William *et al.*, 1997; Barakat *et al.*, 2001)

The objective of this study was to identify leaf rust resistance genes in F2 wheat population by using BSA with SSR markers.

MATERIALS AND METHODS

Plant Material:

Bread wheat somaclonal variant line (S11) developed by Biotechnology laboratory, Crop Science department, Faculty of Agriculture, Alexandria University 2006 from a project (Bio3-001-007 contract No.58), descended from the cultivar Sakha 69, this line characterized by high yield and susceptibility to leaf rust. Spring wheat Pavon 76 (CIMMYT) line resistant to leaf rust, was obtained from Agricultural Research Center, Giza, Egypt (Table 1). They were grown in 2007 growing season at the Experimental Farm of Agriculture Faculty, Alexandria University, Alexandria, Egypt. They were grown in three successive dates at 15 days intervals to overcome differences in the time of flowering. The resistant line was used as male parent for cross with S11 to obtain The F1 seeds. The F1 seeds were grown at the following season 2008 and were selfed to produce F2 seeds. In 2009/2010 growing season, the parents, F1 and F2 plants were evaluated against artificial leaf rust infection (*Puccinia triticina*) at adult stage.

Table 1: Varieties name, country of origin and pedigree for the wheat varieties used in this study.

Wheat genotypes	Pedigree	Developed by	Date of release
Pavon 76	Vicam 71 /7/ (II-19957, Pitic 62 /4/ Kenya 58 / Newthatch /2/ Thatcher /3/ Frontana / Thatcher /5/ Sonora 64) /6/ Siete Cerros 66 /8/ Kalyansona /6/ (II-23584, Ciano 67 /2/ Sonora 64 / Klein Rendidor /5/ (II-8156, (Frontana /2/ Kenya 58 / Newthatch /3/ Norin 10 / Brevor, II-7078) /4/ Gabo 55))	INIFAP, CIMMYT	1976
Sakha 69*	Inia-RL4220 x 7C/yr'S' CM1540-25.65.0S	Egypt	1980

* The variant line S11 descended from Sakha 69 via somaclonal variation and susceptible to leaf rust.

Leaf rust resistance evaluation:

In 2009/2010 growing season, parents and F2 populations were grown under field conditions at the Experimental Farm of Nubaria Agricultural Research Station, Egypt to determine their resistance or susceptibility to *Puccinia triticina*. All plots surrounded by a spreader area, the spreader area was planted with the highly susceptible wheat varieties to the leaf rust pathogen i.e. Sids-1. For the field inoculation, the spreader plants were moistured and dusted with spores powder mixture of the most prevalent leaf rust physiologic races in the area. Dusting was carried out in the early evening (at sunset) before dew formation and when air was still. The inoculation of the plants was carried out at booting stage according to the method suggested by Tervet and Cassell (1951). Data of leaf rust infection types were recorded on the adult stage for each individual plant according to Peterson *et al.*, (1948) as follows:

1 = zero = no visible symptoms.

2 = R = resistant (necrotic areas with or without minute pustules).

3 = MR= moderately resistant (small pustules present surrounded by necrotic halos).

4 = MS= moderately susceptible (medium sized pustules with no necrosis, possible some distinct chlorosis).

5 = S= susceptible (large pustules with no necrosis and little or no chlorosis present).

For the inheritance study, plants with the infection type 1,2 and 3 were considered as resistant infection type. While infection types 4 and 5 were considered as susceptible one, then ratio of resistant to susceptible

plants were determined. Then, goodness of fit to Mendelian ratios was tested by Chi-square test (Steel and Torrie 1960).

DNA extraction:

Genomic DNA was extracted from two-leaf stage seedlings for each parents and F2 plants using CTAB method (Sagahi-Maroof *et al.*, 1984). RNA was removed from the DNA preparation by adding 10 ul of RNAase (10 mg / ml) and then, incubated for 30 min at 37 C . Sample DNA concentration was quantified by using a spectrophotometer (Beckman Du-65).

SSR Analysis:

Thirty three pairs of SSR primers, obtained from Pharmacia Biotech. (Amersham Pharmacia Biotech., UK Limited, HP 79NA, England), were tested in the present experiment, to amplify the templated DNA of the population and the parents. The SSR-PCR method was carried out, according to Roder *et al.*, (1998). Amplification was carried out in 25 µl reaction volumes, containing 1 X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $Mgcl_2$) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany), supplemented with 0.01% gelatin, 0.2 mM of each dNTPs (Pharmacia Biotech, Germany), 250 nM of each primer and 50ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for 1 cycle of 3 min at 94°C, and 45 cycles were performed with 1 min at 94°C, 1 min either 50-60 °C (depending on the individual microsatellite), 2min at 72°C, and a final extension step of 10 min at 72°C. After completion of PCR, samples were immediately cooled to 10°C and stored at 4°C until gel separation. A gel-loading solution (5µl) was added, and 10µl of the total product volume was resolved in 2.5% agarose in 1x TAE buffer for 2 hrs, aside with a 100-pb ladder (Pharmacia, Germany), as the size standard. Gels were stained in ethedium bromide and images were recorded and photographed on gel recommendation system.

Bulked segregant analysis:

Bulked –segregant analysis (Michelmore *et al.*, 1991) was used to target the genomic regions associated with the leaf rust resistance QTLs. Two bulked DNA samples were constructed using equal amounts of DNA from five susceptible and five resistant plants selected based on phenotypic assessments. SSR Primer combinations were then screened on the parents and the two bulked DNA samples, from which some primer combinations revealed bands that were polymorphic, not only between parental genotypes, but also between the pair of the bulked DNA. Based on the evaluations of DNA bulks, individuals of F2 plants were analyzed with cosegregating primers to confirm SSR markers linkage to the leaf rust resistance genes.

Data Analysis:

Goodness of fit to the obtained ratios was calculated for SSR markers by Chi-square test (Steel and Torrie 1960). A simple single marker regression analysis was performed between the SSR markers and the values of leaf rust resistance genes of the F2 plants to determine phenotype: genotype association (Moreno and Gonzales, 1992).

RESULES AND DISCUSSION

This study aims to determine association between microsatellite markers and leaf rust resistance genes in wheat, as an attempt to reach closely linked markers. This can aid combination of Lr genes to provide resistance that is more effective and more durable, using this markers in marker assisted selection of Lr genes in molecular breeding programs (Hiebert *et al.*, 2011), and molecular markers tightly linked to a gene are the starting points for positional cloning of the gene (Martin *et al.*, 1993; Song *et al.*, 1995). For this purpose, the cross resistant line (Pavon 76) X susceptible line (S11) was selected as the source of the segregating population to identify and map SSR markers, linked to the leaf rust resistance genes.

Microsatellite marker analysis

Thirty three microsatellite markers previously mapped in wheat (Röder *et al.*, 1998), were used for polymorphism tests using the two parents and the F2 mapping population. In F2 population, bulked DNA from the F2 individuals, differing in resistance to leaf rust, were used as template for amplification with each primer. SSR primers were screened to identify polymorphism between the parents. Primers that gave clear, distinguishable, and reproducible pattern, were considered for analysis. Out of thirty three pairs of SSR primers two markers only give polymorphsims, the 180 bp fragment, amplified by primer Xgwm 428 was present in the resistant parent, but absent in the susceptible parent S11. Also, this marker was present in the resistant bulked DNA, but not in the susceptible bulked DNA. While primer Xgwm 334 produced polymorphic fragment (180 bp), which it was absent in the resistant parent, but present in the susceptible parent. Moreover, this marker was present in the susceptible bulked DNA, but not in the resistant bulked DNA (Figure 1). These two markers, Xgwm 428 and Xgm334 were further used to check its linkage to the leaf rust resistance genes, using F2 segregating population (Table 2).

Table 2: Description of the two polymorphic microsatellite primers.

Locus	Chromosome	Left primer	Right primer	Repeat	An. temp	Fragment
X gwm428	7D	CGA GGC AGC GAG GAT TT	TTC TCC ACT AGC CCC GC	(GA)22	60°	180 bp
Xgwm334	6A	AAT TTC AAA AAG GAG AGA GA	AAC ATG TGT TTT TAG CTA TC	(GA)19	50°	180 bp

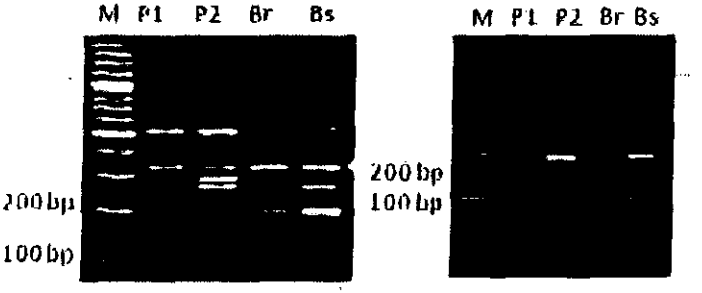

Figure 1: Bulked segregant analysis results with Xgwm428 (left) and Xgwm334 (right) with leaf rust DNA bulks. M: Molecular weight followed by P1 and P2 parents Pavon 76 and S11, respectively. Br, bulk resistance; Bs, bulk susceptible.

The observations showed that the resistant line was resistant to leaf rust and showed no symptoms when exposed to artificial infection. While S11 was susceptible under the artificial infection. F2 population of the cross between the resistant line and S11 segregated to 57 Resistant: 44 Susceptible. This segregation did not fit to the ratio (3R:1S) indicating that the two wheat genotypes did not have one leaf rust resistance gene. However, the segregation ratio of the F2 plants were a good fit to 9:7 ratio, indicating that each of resistant line and S11 have one dominant gene ,which act in a complementary interaction. Many researches supported our, results concerning complementary epistasis. Dyck (1991) attributed the adult plant resistance of Chinese Spring and Sturdy to the interaction of

Lr12 and Lr34. Genetics of leaf rust resistance in spring wheat cultivars Alsen and Norm have been reported (Oelke and Kolmer 2005). Their results indicated that the effective leaf rust resistance in Alsen was due to the interaction of Lr13 and Lr23, with Lr34, and the effective leaf rust resistance in Norm was due to the interaction of Lr13, Lr16, and Lr23 with Lr34. Lal Ahamed and Singh (2003) reported that the segregated population resulted from the cross between Indian wheat variety Kundun and Agra local, indicating that the higher magnitude of epistatic interaction (Complementary epistatic) than the additive and dominance component in the expression of different components of slow rusting at seedling and adult stage. The phenotypic observations were analyzed using goodness of fit to obtained ratios calculated for SSR markers by Chi-square test. SSR marker Xgwm 428, 57 out from the 101 individuals exhibited the amplified polymorphic fragment, while the remaining did not . The ratio did not fit to the expected mendelian ratio 3:1 (x^2 = 17.22, p<0.01). Whereas, 45 from the 101 individuals exhibited the amplified polymorphic fragment (180 bp) by primer Xgwm 334, while the remaining did not. This ratio did not fit to the expected mendelian ratio, 3:1 (x^2 = 15.36, p<0.01) (Table 3). Thus, SSR marker Xgwm 428 appeared to be the result of amplification of genomic DNA, which was linked in coupling to leaf rust resistance gene, designated as Lr D. While SSR marker Xgwm 334 was linked in repulsion to leaf rust resistance gene, designated as LrA. Recently, Datta *et al.*, (2007) found that two complementary recessive genes imparted adult plant leaf rust resistance in DWR 195 (an Indian cultivar), two complementary dominant genes governed the resistance of RAJ 3765 (an Indian cultivar) whereas two were independent dominant.

Table (3): Expected ratio, Chi square and R2 values for SSR markers associated with leaf rust resistance in F2 population (S11x Pavon 76).

Locus	Chromosome	Fragment bp	Expected ratio	X^2	*P* value	R^2 %	*P* value
Xgwm428	7D	180	3:1	17.22	p<0.01	43	<0.001
Xgwm334	6A	180	3:1	15.36	p<0.01	55	<0.001

SSR linkage analysis

The detection of QTLs by the simple regression analysis was developed and showed significant differences between the alternative alleles for SSR markers (Xgwm 428 and Xgwm 334), the calculated R^2 for these markers were 43% (p<0.001) and 55% (p<0.001) respectively (Table 2). The marker which is having a strongest relationship can be judged from its R^2 value which will give the overall percentage of variability of that particular trait that the marker can explain, accordingly it was assumed that these two markers were associated to the quantitative trait loci (QTL), influencing leaf rust

resistance genes. Roder *et al.*, (1998) were mapped Xgwm 428 to chromosome 7DL on long arm whereas, Xgwm 334 mapped to chromosome 6A on short arm. Based on that LrD gene located on 7DL and LrA gene located on 6AS and.

The chromosome 7DS region in wheat is associated with partial resistance to leaf rust, stripe rust , and powdery mildew (Spielmeyer *et al.*, 2005). Marais *et al.*, 2006 found that Lr 56 gene located on chromosome 6 A in *Ae. Sharonensis* , and associated with seedling resistance and linked with Yr 38 .Lin and chen 2009 found that SSR markers: Xgwm 334 (which used in our study) -Xwgp56 Xgwm299 – and Xwgp66r flanking two major QTL , controlling resistance to adult – plant stripe rust , mapped to chromosome 6As, 3BL and 1BL were highly polymorphic in various wheat genotypes, suggesting that these markers are useful in markers- assisted selection . We can deduced that Xgwm334 marker linked to region, located on chromosome 6AS, containing genes controlling resistance to leaf and stripe rust. Kolmer *et al.*, (2010) found that SSR markers barc 57 and barc 321 closely linked to Lr63 on chromosome 3AS in a wheat line. Hiebert *et al.*, (2010) reported that SSR markers cfd71 and cfd23 on a set of 247 wheat lines from diverse origins indicated that these markers can be used to select for the donor segment in most wheat backgrounds. Hiebert *et al.*, (2011) reported that polymorphic SSR markers have been co- inherited with the Lr genes Lr 21, Lr 28, Lr 30, Lr33 and Lr44 in five Near- isogenic lines.

In this study, we obtained two markers, each of them located on different chromosomes, which linked to resistance to leaf rust. Based on that, we can suggest that there are two genes act in complementary interaction, this result agreed with the phenotypic results.We are mapping two Lr genes by using SSR markers, but we need to reach closely linked markers in the next studies. Then, we can combine these Lr genes to provide resistance that is more effective and more durable. Vanzett *et al.*, (2011) found that seedling Lr genes Lr2a, Lr2c, Lr 9, Lr19, Lr 41, and Lr 51 and particularly, Lr 16 showed good levels of resistance against high number of local pathotypes in such a way that cultivars with combinations of complementary Lr genes could show high levels of resistance against leaf rust. They suggested that combinations including seedling resistance genes like Lr16, Lr47, Lr19, Lr41, Lr21, Lr25 and Lr 29, with adult plant resistance gene like Lr34, SV2 and Lr46 will probably provide durable and effective resistance to leaf rust.

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الملخص العربى

تحديد مواقع الصفات الكمية لمقاومة صدأ الورقة في القمح باستخدام الميكروساتيلايت

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[1] قسم الوراثة، كلية الزراعة، جامعة الإسكندرية، مصر.

[2] قسم المحاصيل ، كلية الزراعة، جامعة دمنهور، مصر.

أجريت هذه الدراسة لتحديد وتعيين مواقع الصفات الكمية لمقاومة صدأ الأوراق في عشيرة F2 فى القمح باستخدام تحليل (BSA) مع علامات التسلسل البسيط المتكررة (SSR) . عشيرة ال F2 نتجت من التهجين بين السلالة المختلفة (S11)، الحساسة لصدأ الورقة و Pavon 76 المقاوم لصدأ الورقة. وقد تم تحليل ١٠١ لأفراد العشيرة F2 بالاضافة إلى الآباء باستخدام ثلاثة وثلاثين زوج من دلائل SSR . أظهرت النتائج أن اثنين فقط من أزواج SSR هما Xgwm 428 و Xgwm334 قد أعطوا اختلاف polymorphsim . كلا من العلامات SSR الاثنين قد وجد انهم مرتبطتن بجينات المقاومة لصدأ الورقة LrD و LrA . R^2 المفسر من علامات Xgwm 428 و Xgwm334 هو 43 و 55 ٪، نتيجة للانعزال بين QTL على التوالي. جينات LrD و LrA قد عينت على كروموسوم 7D و 6A على التوالي، والتفاعل بينهم يمكن ان يكون من النوع المكمل.

الكلمات المفتاحية: مقاومة صدأ الورقة، QTL، علامات SSR، *Triticum aestivum*.