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Identification of a microsatellite marker associated with stem rust resistance gene *Sr35* in wheat

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Abstract

A segregating population from the cross '2137'/CRL-Sr35 was made to identify molecular markers linked to the stem rust resistance gene *Sr35* that provides resistance against many devastating races, including QTH and TTKS (or Ug99), in wheat. Ninety-eight F_2 plants were inoculated with a stem rust isolate of race QTH. Infection type and severity were evaluated 10 days after inoculation. Twenty-one microsatellite primer pairs were tested for polymorphism among parental lines and F_2 population. Resistance was conferred by a dominant gene (χ^2=1.01, *P*=0.48). Out of four primer pairs (Xgwm155, Xgwm391, Xgwm497 and Xfca2076) only two markers (i.e. Xgwm391 and Xcfa2076) retained significance at α = 0.05. GWM391 revealed a polymorphic fragment of 200 bp found only in the susceptible bulk and 2137. In contrast, Xcfa2076 amplified polymorphic fragments of 210 bp in the resistant bulk and CRL-Sr35. Using linkage analysis Xgwm391 was found to be relatively close to *Sr35* with a genetic distance of 12.2 cM. Although they have not retained significance at α = 0.05, the markers Xgwm155 and Xgwm497 have genetic distances of 4.6 and 12.1 cM from the *Sr35* gene. While searching for closer marker continues, wheat breeders should be obviously benefited by using these markers in their selection of *Sr35* in their battle against the brutal stem rust race *Ug99*. 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 *Sr35*.

Keywords: *Triticum aestivum*; *Sr35*; stem rust resistance; SSR markers

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important food crops in the world. Stem rust caused by the fungal pathogen *Puccinia graminis* f. sp. *tritici* is a potentially devastating disease of wheat (McCallum et al., 1999), and host plant resistance is the most economical method of controlling it because resistance reduces or eliminates the need for fungicide application (Singh, 2004). Previously, a series of rust resistance genes have been identified in wheat and related species and some of them have been introgressed into wheat (McIntosh and Brown, 1997). However, due to a narrow genetic base and continuously evolving pathogen races, resistant varieties become susceptible to the disease when grown over vast areas (Assefa and Fehrmann, 2004).

The stem rust resistant gene *Sr35* is dominant and is located on the long arm of chromosome 3A (3AL) about 41.5 cM from the centromere and shown 1% recombination with *R2* for red grain color (McIntosh et al., 1995). *Sr35* is considered one of the most highly effective genes against the new African race *Ug99,* also known as TTKS (Jin et al., 2007). This race could reduce wheat yield by up to 71% and could spread and attack many varieties of spring and winter wheat genotypes which are resistant to other strains of the fungus (Pretorius et al., 2000). The *Ug99* is spreading out of Africa. Incorporating *Sr35* into production varieties is therefore urgent. Major gene resistance is short-lived and should be combined with adult stage, minor gene resistance. Durable resistance to rusts and powdery mildew has been supported by minor genes such as *Lr34* for over 50 years (Krattinger et al., 2009). An approach in which major gene resistance, conferred by genes such as *Sr35*, is

combined with minor gene resistance should be effective against the threat of *Ug99*.

Evaluation of stem rust resistance is time consuming, laborious and costly because the inheritance of resistance is complicated by the recessive nature of some of the resistance genes and the potential effect on phenotype of other stem rust resistance genes present in genetic backgrounds (Spielmeyer et al., 2003). Closely linked markers provide an alternative means for the selection of important genes in breeding programs and, in the case of disease resistance, this can be done in the absence of pathogens (Park et al., 2002). A molecular marker for *Sr35* is badly needed for breeding wheat that is resistant to the emerging severe pathogen race *Ug99*.

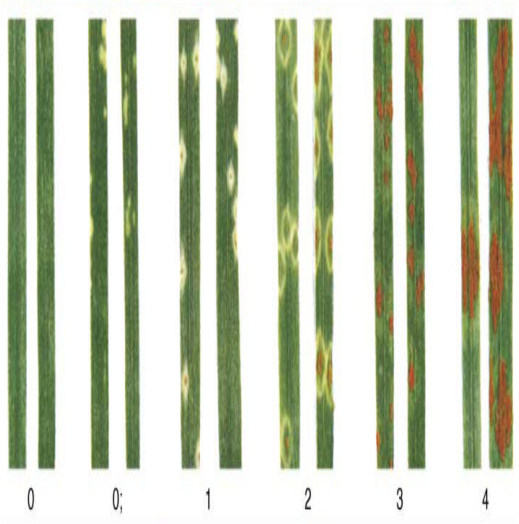

Fig 1. Infection types of *Puccinia graminis* Sp. *tritici* on the wheat seedlings [(adapted from Stakman et al., 1962), where 0= invisible symptoms (immune), 0;= hypersensitive necrotic or chlorotic flecks without uredinia (highly resistant), 1= small uredinia surrounded by necrosis (resistant), 2= small to medium sized uredinia surrounded by necrotic areas (moderately resistant), 3= medium to large sized uredinia without chlorosis (susceptible), and 4= large sized uredinia without chlorosis (highly susceptible)].

Microsatellite markers, also called simple sequence repeats (SSRs), have been developed to exploit repeated DNA sequence variation and have become popular with wheat geneticists. These markers are based on a 1 to 6 nucleotide core element, such as (GT)*n* or (CT)*n*, that is tandemly repeated from two to tens of times (Peng et al.,1999). A different "allele" occurs at a microsatellite locus as a result of changes in the number of times the core element is repeated, altering the length of the repeated region. SSR markers are co-dominant and locus specific (Sourdille et al., 2004). Their co-dominant nature facilitates differentiation of homozygotes and heterozygotes in early generations of segregating populations (Röder et al., 1998a). This feature can significantly speed up the breeding process and improve selection efficiency (Zhou et al., 2003). Our objective is to identify a DNA marker for the stem rust resistance gene *Sr35* in F_2 population of wheat.

Materials and methods

Stem rust evaluation

For genetic mapping of stem rust resistance gene *Sr35*, F_1 seeds were developed by crossing the susceptible hard winter wheat cultivar '2137' (PI 592444) (Sears et. al., 1997) to an unreleased resistant spring wheat germplasm CRL-Sr35 (Yue Jin, Cereal Disease Laboratory, St. Paul, Minnesota, USA, Personal communication), which has resistance that was transferred from *Triticum monococcum* (McIntosh et. al., 1984), in fall 2004 and advanced in the greenhouse. The F_2 seeds were grown and about 0.3-0.4 g of fresh leaf tissue was collected from each plant and frozen in liquid nitrogen for DNA extraction. Two-weeks-old seedlings of parental and 98 lines of F_2 population were inoculated at the 3-leaf stage with a stem rust isolate of race QTH that was avirulent on CRL-Sr35 but virulent on 2137.

Briefly, the gelatin capsules that contained the race were shocked at 42°C and a 'dark tea' of spores in approximately 50 mL of Soltrol 170 oil was sprayed at 7 psi 6 inches above the plants, directed at the leaves, covering each tray 3 or 4 times. Plants were allowed to dry for 40 minutes. Mist was turned on at 3 PM and continued until 8 AM the following day. After 20 min of continuous misting, the cycle was set at 40 sec on every 20 min. After 18 h of misting in the dark, lights were turned on. The chambers were opened half way for two hours. Then the plants were moved to the greenhouse. Disease reaction was determined 10 days after inoculation based on the infection types using a 0 - 4 scale developed by Stakman et al. (1962; Fig. 1).

DNA extraction

Genomic DNA was extracted using the plant genomic DNA isolation reagent DNAzol^{TM}ES (Molecular Research Center, Inc., Cincinnati, Ohio, USA), with some modification. About 0.3 to 0.4 g of fresh leaf tissue was put into a microfuge tube and ground in liquid nitrogen to a fine powder with a plastic pestle. DNAzole solution (700µL) was then added and mixed for at least 5 min. Chloroform (700µL) was added to the solution, vortexed for 20 sec, incubated for 5 min and centrifuged at 10,000g for 5 min. The supernatant

Table 1. F_2 Segregation pattern of reaction to stem rust isolate QTH in the cross 2137/CRL-Sr35.

Category	Observed number	Expected number	χ^2
	(O)	(E)	$(O - E)^2/E$
RR[§] + Rr	69	73.5	0.276
Rr	29	24.5	0.827
			1.01[¶]

[§]RR = homozygous resistant, Rr = heterozygous resistant, and rr = homozygous susceptible F_2 plants
[¶]P=0.48

(500µL) was transferred into a new microfuge tube and total nucleic acids were precipitated with two volumes of cold ethanol. The tubes were chilled at -20 ºC for 30 min and then centrifuged at 5,000g (4ºC) for 5 min. The supernatant was carefully discarded and the DNA pellet was resuspended in 140µL TE buffer (10mM Tris pH 8.0, 1Mm EDTA) and 1400µl DNAzole:Ethanol (4:3) solution and the tubes were incubated for 5 min. After that the tubes were centrifuged at 5,000g for 5 min. The supernatant was discarded and the DNA pellet was washed twice in 75% ethanol and dried by air for 30 min. The DNA was resuspended in 100µL 8mM NaOH, and the pH was adjusted to 8.0 by adding 10 µL of 0.1M HEPES. Insoluble material was removed by centrifugation at 12,000g.

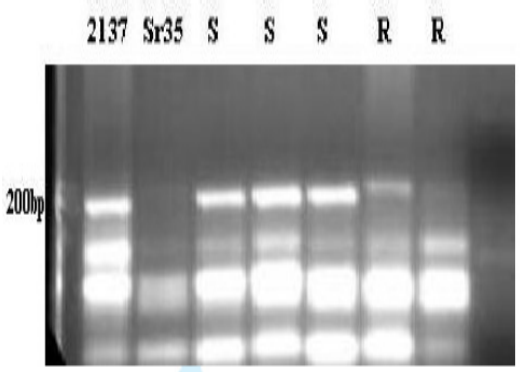

Fig 2. PCR amplification pattern of genomic DNA using SSR primer set gwm 391 (2137, Sr35, R and S: cultivar 2137, germplasm line CRL-Sr35, and resistant and susceptible F_2 phenotypes, respectively).

Microsatellite marker analysis

Microsatellite markers previously mapped in wheat chromosome 3A and surrounding the chromosomal regions that contain *Sr35* were used for polymorphism tests using the two parents of the mapping population. Polymorphic markers were chosen to genotype DNA samples of the mapping population. The SSR markers used in this study included Xgwm2, Xgwm5, Xgwm30, Xgwm32, Xgwm114, Xgwm133, Xgwm155, Xgwm162, Xgwm369, Xgwm391, Xgwm480, Xgwm497, Xgwm666 (Röder et al. 1998a), Xbarc25, Xbarc45, Xbarc179, Xbarc284 and Xbarc314 (Song et al,. 2005) Xcfd2 and Xcfa2076 and Xcfa2193 (Somers et al., 2004). Primer sequences were obtained from www.graingenes.org. The PCR reactions were performed as described by Röder *et al.* (1998b) in a volume of 20 µL in MJ Research PTC-200 Thermal Cycler. The reaction mixture contained 250 nM of each primer, 0.2 mM of each deoxynucleotide, 1.5 mM $MgCl_2$, 1 unit Taq polymerase (Invitrogen), 80-100 ng of template DNA, 10 mM Tris-HCl, pH 8.3 and 50mM KCl. For GWM and BARC markers, after 3 min at 94 ºC, 35 cycles were performed with 1 min at 94ºC, 1 min at either 50, 52, 55 or 60 ºC (depending on primer annealing temperature), 2 min at 72 ºC and final extension step of 5 min at 72ºC, while for CFA and CFD 5 min at 94°C; 30 cycles (94°C for 30 sec; 60°C for 30 sec; 72°C for 30 sec); 10 min at 72°C.

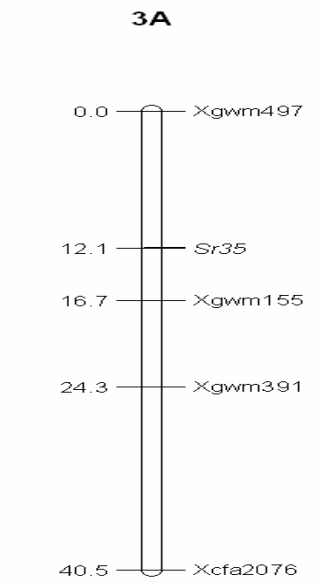

Fig 3. Linkage map of wheat chromosome 3A containing stem rust resistance gene, *Sr35*. Genetic distances are given in cM.

To detect the polymorphism between parents, 9 µL from each PCR product were denatured for 5 min at 95ºC, after adding 3 µL loading buffer (containing 98% formamide, 10mM EDTA/L pH 8.0, 0.25% Xylene cyanol), separated on a 3.5% Metaphor™ agarose gel and electrophoresed at 60 V for 2 h.

Table 2. Characteristics of the screened microsatellite markers.

Marker	Primer sequences	Ann. Tem. (ºC)	Product size in 2137 (bp)	Product size in CRL-Sr35
Xbarc314	5'CTGTGGAAACCAATAAAAACAA3' 5'GTGCGCGAATAACTACAAGAAA3'	50	260, 280	200, 260
Xcfa2076	5' CGAAAAACCATGATCGACAG 3' 5' ACCTGTCCAGCTAGCCTCCA 3'	60	200	210
Xcfa2193	5' ACATGTGATGTGCGGTCATT 3' 5' TCCTCAGAACCCCATTCTTG 3'	60	200	200, 210
Xgwm155	5' CAATCATTTCCCCCTCCC 3' 5' AATCATTGGAAATCCATATGCC 3'	60	100,110,130 160,170,220	100, 160, 170
Xgwm162	5' AGTGGATCGACAAGGCTCTG 3' 5' AGAAGAAGCAAAGCCTTCCC 3'	60	190, 210	200, 220
Xgwm391	5' ATAGCGAAGTCTCCCTACTCCA 3' 5' ATGTGCATGTCGGACGC 3'	55	180, 190, 200	180, 190
Xgwm480	5' TGCTGCTACTTGTACAGAGGAC 3' 5' CCGAATTGTCCGCCATAG 3'	60	100, 120, 200	100, 120, 130, 190
Xgwm497	5' GTAGTGAAGACAAGGGCATT 3' 5' CCGAAAGTTGGGTGATATAC 3'	55	170, 190	160, 170, 210
Xgwm666	5' GCACCCACATCTTCGACC 3' 5' TGCTGCTGGTCTCTGTGC 3'	60	105	105, 110, 115, 120

The bands were visualized after ethidium bromide staining (10mg mL^{-1}) and photographed using a BioRad Chemi Doc XRS Imaging station (Tika et al., 2004). Presence or absence of the markers was recorded for each individual line. Chi-square analysis was used to test the significant marker-trait association for each of these markers.

Linkage analysis

Mapmaker/Exp 3.0b (Lander et al., 1987) was used to estimate the genetic distance between the SSR markers and the *Sr35* gene.

Results

Inheritance of stem rust resistance

Screening of the F2 individuals revealed that 69 were resistant and 29 were susceptible. The result was subjected to chi-square analysis for a 3:1 ratio. The chi-square values showed that the F2 progeny clearly fit a 3:1 ratio (χ^2 =1.01, *P*=0.48; Table 1), thus indicating that the stem rust resistance was controlled by a single dominant gene. These results agreed with those found by McIntosh et al. (1984).

Microsatellite polymorphism between the parental genotypes

Out of 21 SSR markers used in this study, only nine primer pairs generated polymorphism between the parents (Table 2). Each of these markers was used to screen DNA bulks of nine susceptible and nine resistant F_2 plants. SSR markers Xgwm155, Xgwm391, Xgwm497 and Xcfa2076 amplified polymorphic bands. Significant marker-trait association for each of these markers was conducted by genotyping all the F_2 population and analyzed by Chi-square. However, after genotyping the whole population, only two markers (i.e. Xgwm391 and Xcfa2076) retained significance at $\alpha = 0.05$ (Table 3). GWM391 revealed a polymorphic fragment of 200 bp found only in the susceptible bulk and susceptible parent (Fig. 2). Chi-square analysis revealed that the gwm391 marker was associated with susceptibility (χ^2 =3.135; *P*=0.1), with a genetic distance of 12.2 cM (Fig. 3). Primer pair Xcfa2076 amplified a polymorphic fragment of 210 bp in the resistant bulk and CRL-Sr35, 200 bp bands in the susceptible bulk and 2137. Chi-square analysis revealed that the Xcfa2076 marker was associated with *Sr35* and no significant deviation from the expected ratio was observed (χ^2=0.78; *P*=0.4). This marker was found to be far from the *Sr35* gene with a genetic distance of 28.4 cM (Fig. 3). Although they have not retained significance at $\alpha = 0.05$, the markers Xgwm155 and Xgwm497 have genetic distances of 4.6 and 12.1 cM from the *Sr35* gene (Fig. 3).

Discussion

The resistance gene *Sr35* is located 41.5 cM from the centromere and has shown 1% recombination with *R2* for red grain color (McIntosh et al., 1995). Red grain color is a classical marker for seed dormancy in wheat (Adlam and Flintham, 1999). Parker et al. (1998) found that the RFLP marker Xbcd828-3A was significantly associated with flour color. Eryman et al. (2004) predicted that *Sr35* is located in the interval between RFLP markers Xbcd131 and Xwia807. RFLP markers Xabc174 and Xbcd131 mapped within 5 cM of the wheat R loci (Adlam and Flintham, 1999). In this study only four microsatellite markers (Xgwm155, Xgwm391, Xgwm497 and Xcfa2076) amplified polymorphic bands between parents and F_2 bulks. Somers et al. (2004) located Xgwm497 and Xcfa2076 on 3AL at about 27 and 51.7 cM from the centromere,

Table 3. Microsatellite markers linked to stem rust resistance gene *Sr35*

Marker	No. of F_2	Observed number (O)		Expected number (E)	χ^2 $(O-E)^2/E$
gwm391	98	RR + Rr[§]	61	69	0.928
		rr	37	29	2.206
					$\chi^2 = 3.135$[¶]
Xcfa2076	98	RR + Rr	73	69	0.232
		rr	25	29	0.552
					$\chi^2 = 0.78$[±]

[§]RR = homozygous resistant, Rr = heterozygous resistant, and rr = homozygous susceptible F_2 plants
[¶]P=0.1
[±]P=0.4

respectively. According to the wheat microsatellite map (Röder et al., 1998a) the microsatellite marker Xgwm391 is proximal to the RFLP markers Xabc172 and tam63 on 3AL. Our study revealed two polymorphic microsatellite markers between the resistant and susceptible parents and in the F_2 population. The 200 bp band generated by Xcfa2076 is associated with the resistant allele but, ironically, was found to be far from *Sr35*. The microsatellite marker gwm391 revealed a 200 bp polymorphic band associated only with the susceptible allele and can be used for detection of the susceptible plants that do not carry this gene (Fig. 1). Although they have not retained significance at $\alpha = 0.05$, the markers Xgwm155 and Xgwm497 have genetic distances of 4.6 and 12.1 cM from the *Sr35* gene (Fig. 3).Although these four markers still are relatively distant from *Sr35*, they are currently the closest known markers to the gene. In our study, 94.5% of the F_2 plants that have the Xcfa2076 marker were resistant, while 78.4% of the F_2 plants that have the Xgwm391 marker were susceptible. While searching for closer marker continues, wheat breeders should be obviously benefited by using these markers in their selection of *Sr35* in their battle against the brutal stem rust race *Ug99*. 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 *Sr35*.

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