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Preliminary Studies on Microsatellite Marker Analysis of Resistance to Common Bunt in several Wheat Genotypes (*Triticum aestivum* L.)

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Abstract

In order to use markers for the selection of wheat resistance to the common bunt, there were tested certain wheat microsatellite markers (SSRs) on certain local accessions of bread wheat. A number of eleven SSR markers were tested on parental forms (five lines of bunt resistant wheat - *Bt5*, *Bt8*, *Bt10*, *Bt11* genes and a number of eight cultivars and lines susceptible to common bunt. From the eleven tested markers, the *Xgwm633* marker and *Xgwm114* marker identified polymorphism on parental forms. Relying on co-segregation method, the *Xgwm633* marker revealed a polymorphic fragment of 200 bp on the resistant wheat line '99419G4-1A/1-1' and a 230 bp fragment on the susceptible cultivar 'Glosa'. So the fragment of 200 bp obtained with this marker could be assigned to some common bunt resistant genes. The *Xgwm114* marker revealed a polymorphic fragment of 120 bp on resistant wheat line '00274G2-31', harboring the *Bt11* resistant gene, and two fragments of 160 bp and 180 bp on susceptible cultivar 'Glosa'. So the fragment of 120 bp obtained with this marker could be assigned to *Bt11* resistant gene. That result was also confirmed by BSA (Bulked Segregant Analysis) method. The linkage relationship between markers and resistant genes was evaluated on the F_2 populations resulted from the cross between '99419G4-1A/1-1' and '00274G2-31' the wheat resistant lines with the susceptible cultivar ('Glosa'). The value of recombination between the *Xgwm633* marker and a common bunt resistant gene was evaluated at 32 cM. A smaller value of recombination has been estimated between the marker *Xgwm114* and the resistant gene (8 cM).

Keywords: SSRs markers, common bunt, wheat, resistance, BSA

Introduction

Wheat common bunt is a disease caused by *Tilletia carries* and *T. foetida* species, which determines important production losses in wheat cultures, affecting also the quality of the harvested seeds. The seeds treatment with fungicides represents the most frequent means to control this disease (Nagy and Moldovan, 2007). The performed tests show that currently there are resistance sources at the species cultivated now in Europe (Huber and Buerstmayr, 2006). Introducing these resistance sources in the cultivated varieties through the breeding programs is an important control strategy, especially for the organic agriculture. The availability of the microsatellite markers (SSRs) transmitted in linkage with some common bunt resistance genes could accelerate the process of breeding and selection. Until now, some RAPD molecular markers for some common bunt resistance genes have been identified (Laroche *et al*., 2000), He and Hughes (2003), but also SSRs markers (Fofana *et al*., 2008; Wang *et al.,* 2009). Microsatellite markers (SSRs) are the most frequently used for wheat owing mainly to their high polymorphism (Ganal and Röder, 2007). Using the markers in the marker assisted selection process involves a tight linkage with the resistance gene. As this analysis Bulked Segregant Analysis (BSA) described by Michelmore *et al*. (1991) represents a quick method to identify markers tightly linked to the genes affecting the character of interest in the segregant populations (Botez *et al*., 2009; Sestras *et al*., 2009).

The goal of the analyses carried out was identifying the markers tightly linked to the common bunt resistance genes, detecting their presence in some Romanian wheat genotypes. In order to do this, SSRs markers were tested; they were selected according to the information existing in specialized literature regarding their linkage or association to some common bunt resistance genes.

Material and methods

Plant material

In order to test the polymorphism for common bunt resistance we have used a number of 5 common bunt resistant genotypes (Tab. 1). We have also tested a number of 8 genotypes (lines and cultivars) sensitive to this disease: 'Farmec', 'Delabrad', 'F96869G1-108', 'Glosa', 'Boema', 'Jiana', 'Crina' and 'Dropia'. These lines and cultivars come from NARDI-National Agricultural Research and Development Institute Fundulea, Romania. The analysis with microsatellite primers was performed both for the parental forms and for two F_2 populations. The segregant populations were obtained from crossing the resistant line '99419G4-1A/1-1' to the sensitive cultivar 'Glosa' and from crossing the resistant line '00274G2-31' to the cul-

Tab. 1. Resistant parental wheat genotypes (lines) used in analyses, resistance source

Genotype	Genealogy	Gene (Bt)
'99419G4-1A/1-1'	Colonias/'Bucur'	Unknown
'00274G2-31'	'Dropia'*2/*Bt5*//'Delabrad'	*Bt5*
'00281G2-11'	'Dropia'/*Xt11*//'Delabrad'	*Bt11*
'00399G2-11'	'F7022W1'/5/ PI178383/'Iulia'//'Aura'/3/ FL80/4/'Dropia'/6/'Delabrad'	*Bt8* or *Bt10*
'00450G1-1'	'Dropia'/*Xt11*//'Delabrad'/3/ 'F93122G6-209'	*Bt11*

tivar 'Glosa'. The '99419G4-1A/1-1' wheat line represents an important resistance source, and one of the genitors of this line is the landrace cultivar Colonias which the resistance comes from. However, the resistance genes coming from Colonias have not yet been identified. The 'Glosa' wheat variety was characterized by the breeder as very sensitive both to natural and artificial infection with common bunt.

The resistance testing of the F_2 plants was carried out by artificial infection in field conditions, by the mixture of seeds with *Tilletia (T. carries* and *T. foetida)* spores.

The DNA extraction was performed from leaves, fresh or frozen at - 80 °C temperature, using the protocol elaborated by Lodhi *et al*. (1994), based on the CTAB method (Cetyltrimethyl Ammonium Bromide). After the extraction, the DNA was re-suspended in the TE (Tris-EDTA) solution. After the dissolution of the DNA pellet, the concentrations and purities of the DNA solution were determined using the Eppendorf biophotometer, and then it was diluted to a concentration of 30 ng/µl, which was used in PCR reactions.

The Bulked Segregant Analysis (BSA) was performed at the two F_2 populations ('99419G4-1A/1-1'x'Glosa' and '00274G2-31'x'Glosa'). Equal quantities (2,5 µg) of DNA from each individual F_2 plant, phenotipically characterized as resistant respectively sensitive (5-6 plants/bulk), chosen at random, were mixed in order to create resistant bulks (Rb) and sensitive bulks (Sb), (Michelmore *et al*., 1991).

Used Microsatellite markers (SSRs)

A set of microsatellite markers were tested for the polymorphism, markers located on different wheat chromosomes in regions where some resistance genes (*Bt*) are also located, present in the analyzed germplasm (Tab. 2). The set of primers for markers were synthesized at Microsynth Company, Switzerland, and includes 11 pairs of primers. The sequence of primers was obtained from the USDA ARS GrainGenes database (www.graingenes.org) and from the published articles presenting the chromosome maps of wheat (Röder *et al*., 1998; Somers *et al*., 2004).

The PCR amplification reactions were performed in a volume of 25 µl reaction mixture containing: 1X of 10XDreamTaq™Green Buffer (Fermentas) with 20mM $MgCl_2$, 200µM dNTPs mix, 0,2 µM forward primer, 0,2 µM reverse primer, 1 unit Dream*Taq*™ DNA polymerase (Fermentas), ADN (60 ng)/reaction as the matrix chain. The amplification program was the following: the first step of denaturation at 94°C for 3 minutes, 35 cycles with the following steps: the first step at 94°C for 1 minute, 50-60°C - attaching temperature (depending on the primer) for 1 minute and the last step at 72°C for 2 minutes, and the final extension at 72°C for 2 minutes.

The electrophoresis of PCR products. The amplification products were migrated through electrophoresis in agarose gel 1,4%, in 0,5XTBE (Tris-Borate-EDTA) buffer at 80 V for approximately 1.5 h. In order to determine the size of the amplified fragments we used ladder of 100 pb (Fermentas). The coloring of the agarose gel electrophoresis was achieved with ethidium bromide, and then they were visualized under UV light and photographed. After that, in order to establish the precise dimension of the initially visualized fragments in the agarose gel and of the polymorphism, the denaturing polyacrylamide gel electrophoresis

Tab. 2. Used microsatellite markers (SSRs)

Marker	Location/ Chromosome	Character	Bibliography
Xgwm818	*1B*	Flank. QTL	Fofana *et al.,* 2008
Xgwm264	*1B, 3B*	SSR for Bt12	Wang *et al.,* 2009
Xgwm374	*1B, 2B*	SSR for Bt12	Wang *et. al.*, 2009
Xgwm403	*1B, 2B, 3A, 3B*	Flank. QTL	Fofana *et al.,* 2008
Xgwm259	*1B*	Gene from Aegilops	Galaev *et al.,* 2006
Xbarc128	*1B, 2B*	SSR codom.	Wang *et al.,* 2009
Xgwm469	*1B, 5D, 6D, 6DS*	(19.3 cM) to Bt10	Menzies *et al.,* 2006
Xgwm749	*6D*	Bt10	Menzies *et al.,* 2006
Xgwm63	*7A*	Flank. QTL	Fofana *et al.,* 2008
Xgwm633	*7A*	Flank. QTL	Fofana *et al.,* 2008
Xgwm114	*3B*	for Bt11	Goates *et al.,* 2009

6% (acrylamide, bisacrylamide, urea, 0.5XTBE) has been used by migration at 250 V for 2 hours in 0,5XTBE buffer. Part of the PCR products migrated in the polyacrylamide gel were detected by staining with $AgNO_3$ (silver staining), according to the protocol described by Bassam *et al*. (1991). It was used 0.3 µg of O´ Range Ruler™ (Fermentas), (20 pb) as ladder of molecular dimension.

Results and discussions

Concerning the transmission of resistance in F_2 generation, at individuals obtained from the cross '99419G4-1A/1-1'x'Glosa', the χ^2 test shows that the F_2 segregation ratio for the tracked character was of 3:1, indicating the existence of a unique locus which controls the common bunt

resistance present in line '99419G4-1A/1-1' (χ^2=0,24; P=0,62). This segregation ratio was also obtained at '00274G2-31'x'Glosa' population (χ^2=1,7; P=0,19).

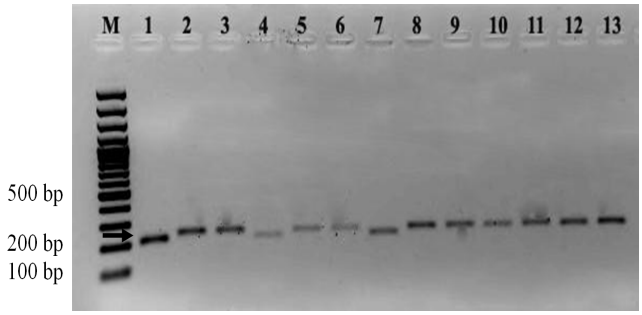

Fig. 1. Agarose gel electrophoresis of the products of amplification obtained with the *Xgwm633* marker at resistant parental forms 1-'99419G4-1A/1', 2-'00274G2-31', 3-'00281G2-11', 4-'00399G2-11', 5-'00450G1-1', and sensitive parental forms 6-'Farmec', 7-'Delabrad', 8-'F96869G1-108', 9-'Glosa', 10-'Boema', 11-'Jiana', 12-'Crina', 13-'Dropia', M-ladder 100 bp

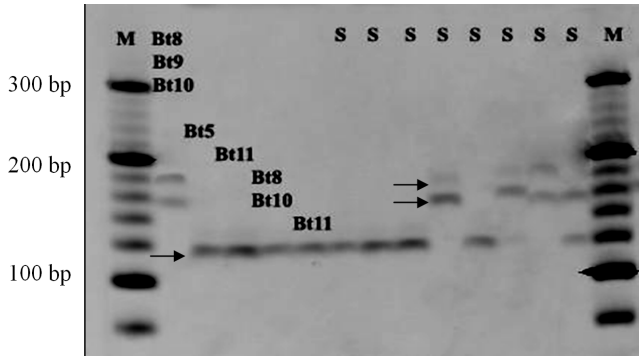

Fig. 2. Polyacrylamide gel electrophoresis of products of amplification obtained with *Xgwm114* marker at the resistant parental forms with resistance genes (*Bt5, Bt8, Bt11*) and at the 8 sensitive genotypes, M-ladder of 20 bp

Assigning the SSRs markers to the common bunt resistance genes according to the cosegregation

The preliminary analysis of the 11 microsatellite markers (Tab. 1) shows that only two markers (*Xgwm633* and *Xgwm114*) have identified polymorphic fragments between the parental genotypes of wheat, common bunt resistant and sensitive. The *Xgwm633* marker highlighted a polymorphic fragment of 200 bp at the resistant line '99419G4-1A/1-1', fragment which was not present in any sensitive parental form, and a fragment of 230 bp in all 8 sensitive genotypes (Fig. 1).

It seems that the fragment of 200 bp marks within the '99419G4-1A/1' line an unknown common bunt resistance gene, which is absent in all the sensitive parental forms, and also in the other resistant wheat lines. According to the observation of Fofana *et al.* (2008), this marker is located near a QTL for common bunt resistance; it is suggested that in this case also the fragment of 200 bp marks one of the QTL determining the resistance.

The *Xgwm114* marker is specific for *Bt11* gene and marked out a polymorphic fragment of approximately 120 pb at 4 out of the 5 resistant parental genotypes; this resistance fragment was also identified by Cichy and Goates, (2009) at genotypes with *Bt11* gene. In the present analyses, the polymorphic fragment of 120 bp for resistance has been identified at the two genotypes with the *Bt11* gene ('00281G2-11' and '00450G1-1'), gene which is located on the chromosome 3B, but also on 3 out of the 8 sensitive genotypes (Fig. 2). The fact that the resistance fragment was amplified not only in the known lines with *Bt11* gene, as expected, but also in the genotypes bearer of some other resistance genes (*Bt5*, *Bt8, Bt10*) could indicate that these genotypes carry, together with the mentioned genes, the *Bt11* gene too. This fact is possible as these genotypes have high resistance to common bunt, *Bt11* gene being one of the genes providing the avirulence towards all the races of bunt.

Assigning the SSRs markers to the common bunt resistance genes according to the Bulked Segregant Analysis (BSA) method

The results concerning the segregation for resistance and the two polymorphic SSR markers (*Xgwm633* and *Xgwm114*) in F_2 generation are shown in Tab. 3.

The segregant '99419G4-1A/1-1' x 'Glosa' population, formed of a number of 51 plants, was genotypically tested with this marker. The *Xgwm633* marker, cited by Fofana *et al*. (2008) as being linked to a QTL for common bunt resistance, in our analyses marked out the two fragments of 200 bp and 230 bp (Fig. 3). The BSA analysis for the *Xgwm633* marker confirms the presence of the two polymorphic fragments, that of 200 bp for the DNA mixture coming from the resistant plants, and that of 230 bp coming from the sensitive plants (Fig. 3). Thus we have the confirmation that the *Xgwm633* marker, by the fragment of 200 bp, marks a resistance allele which is linked with in the coupling phase, the marker being located at a distance of 32 cM from the resistance gene. The relatively high estimated value of recombination between the *Xgwm633*

Tab. 3. F_2 plant segregation for resistance and polymorphic markers

Xgwm633 marker	'99419G4-1A/1-1' x 'Glosa' population			
	A-B-	A-Bb	aa-B-	aabb
	22	7	14	5

Xgwm114 marker	'00274G2-31' x 'Glosa' population			
	A-B-	A-bB	aaBb	aabb
	14	20	6	12

Testing the '99419G4-1A/1-1'x 'Glosa' population with *Xgwm633* marker

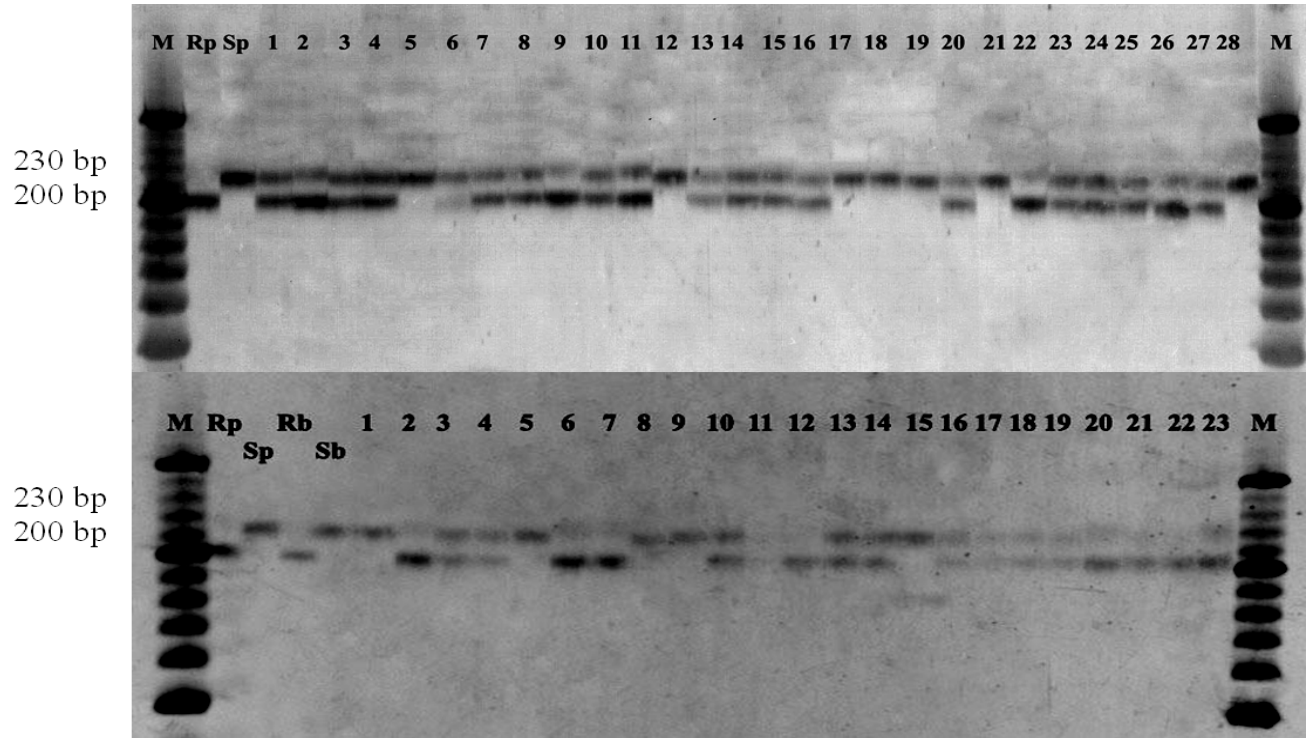

Fig. 3. Polyacrylamide gel electrophoresis of products of amplification obtained with marker *Xgwm633* at F_2 plants from the '99419G4-1A/1-1'x 'Glosa' population: Rp-resistant parent, Sp-sensitive parent, Rb-resistant bulk, Sb-sensitive bulk, 51 F_2 segregant plants (1-28 and 1-23), M - ladder 20 bp

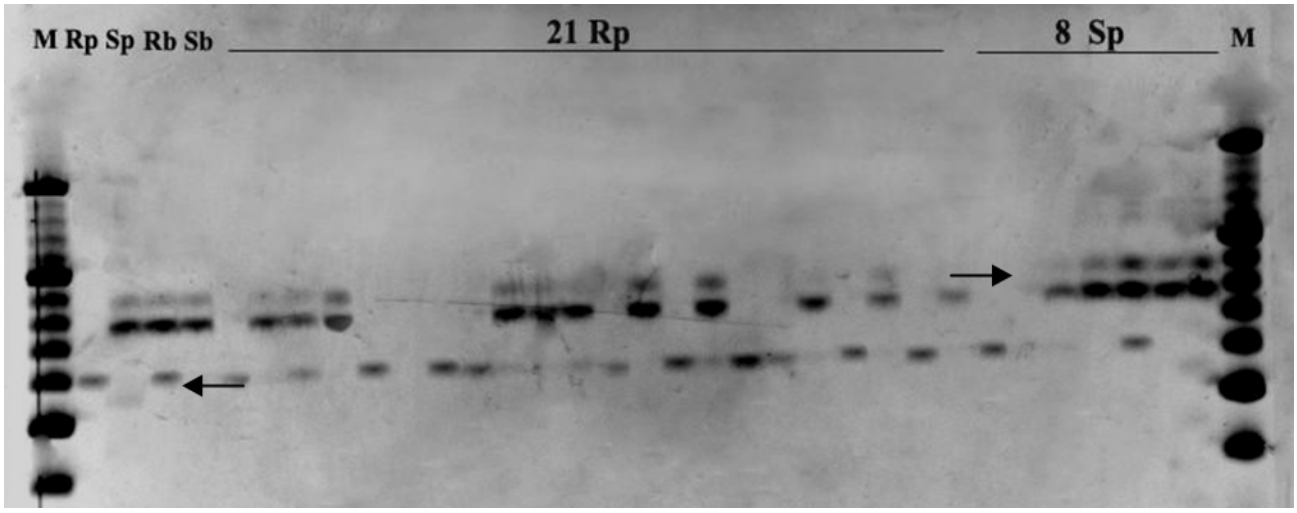

Fig. 4. Polyacrylamide gel electrophoresis of products of amplification obtained with marker *Xgwm*114 at F2 plants from the '00274G2-31' x 'Glosa' population: Rp-resistant parent, Sp-sensitive parent, Rb-resistant bulk, Sb-sensitive bulk, Rp-F2 resistant plants, Sp-F2 sensitive plants. M - ladder 20 bp

marker and the common bunt resistance gene might be because the marked gene actually belongs to a QTL involved in quantitative resistance to common bunt, the resistance under polygenic control.

Testing the '00274G2-31' x 'Glosa' population with *Xgwm114* marker.

This F_2 population consisted of a 52 plants. The Xgwm114 marker showed at the two bulks the same polymorphic fragments as at the parental forms. However, at the bulk obtained from resistant plants, in addition to the fragment of 120 bp specific to the parental resistant lines, the two fragments of 160 bp and 180 bp, specific to the susceptible parental form, were observed (Fig. 4). This may be due to the fact that among the plants considered resistant, one plant carrying the susceptible allele was selected, but not manifesting the disease. It should be noted that the fragment of 120 bp specific to the resistant lines has also been reported by Cichy and Goates (2009); Cichy (personal communication, April 20, 2010). But at the

susceptible genotype they have found a single fragment of 110 bp.

Based on the analysis of recombination values the distance between the marker and the resistance gene was estimated to be 8 cM. In order to calculate the recombination value the frequency of the double recessive offspring estimating the parental type gametes frequency was used. It was considered the fact that the marker and the gene for resistance are linked in a coupling phase. The present analysis was performed apart from the codominant inheritance of the molecular marker.

The smaller value of recombination evaluated between *Xgwm114* marker and the resistant gene (8 cM) made from this marker a good candidate in the selection program for a qualitative resistance of wheat to common bunt.

Conclusions

Among the eleven microsatelite marker (SSRs) tested only two (*Xgwm633* and *Xgwm114*) showed molecular polymorphism between resistant and susceptible wheat genotypes to common bunt.

Relying on the two methods of analysis used (cosegregation and BSA) the two markers were assigned to the bunt resistance genes.

The microsatellite marker *Xgwm633* seems to be a marker for a resistance QTL, the recombination value was estimated to 32 cM. The enough large value of recombination evaluated between *Xgwm633* marker and a common bunt resistant gene (32 cM) could be due the fact that the gene belongs to a QTL implied in quantitative resistance to common bunt.

The microsatellite marker *Xgwm114* marker proved to be linked at 8 cM with the *Bt11* resistance gene. This marker indicated the presence of the *Bt11* gene among wheat genotypes known as carriers of other resistance genes (like *Bt5, Bt8* and *Bt10*). The smaller value of recombination evaluated between *Xgwm114* marker and resistant gene (8 cM) made from this marker a good candidate in the selection program for a qualitative resistance of wheat to common bunt.

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