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ORIGINAL PAPER

Genetic mapping of the stem rust (*Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn) resistance gene *Sr13* in wheat (*Triticum aestivum* L.)

Belayneh Admassu · Dragan Perovic · Wolfgang Friedt · Frank Ordon

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Abstract *Puccinia graminis* f. sp. *tritici*, the causative agent of stem rust in wheat, is known for its high virulence variability and ability to evolve new virulence to resistance genes. Thus, pyramiding of several resistance genes in a single line is the best strategy for a sustainable control of wheat stem rust. *Sr13* is one of the few resistance genes that are effective against wide ranging *P. graminis* f. sp. *tritici* races, including the pestilent race Ug99. Its effectiveness to Ug99 makes it a valuable source for resistance to stem rust. Molecular markers play a pivotal role in the genetic characterization of the new sources of resistance as well as in stacking two or more resistance genes in a single line. Therefore, the aim of this study was to develop molecular markers for *Sr13* facilitating efficient pyramiding of Sr genes. Based on the 158 F_2 individuals derived from a cross of Khapstein/9*LMPG × Morocco and SSR analyses, the *Sr13* locus was mapped on chromosome 6A of wheat, and a genetic map comprising about 90 cM was constructed with the closest marker barc37 being located 4.0 cM distally of *Sr13*. Of the nine mapped markers, barc37 amplified an allele specific for the presence of *Sr13* as shown by testing different cultivars and breeding lines. These newly developed markers will increase the efficiency of incorporating *Sr13* into cultivars that are widely adopted, but susceptible to hazardous Ug99 and/or assist for the development of new elite lines that are resistant to Ug99.

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B. Admassu
Plant Protection Research Center, Ethiopian Institute of Agricultural Research, P. O. Box 37, Ambo, Ethiopia

B. Admassu (✉) · D. Perovic · F. Ordon
Julius Kuehn-Institute, Federal Research Institute for Cultivated Plants (JKI), Institute for Resistance Research and Stress Tolerance, Erwin-Baur-Str. 27, 06484 Quedlinburg, Germany
e-mail: belayneh.admassu@jki.bund.de

W. Friedt
Institute of Crop Science and Plant Breeding I, Justus-Liebig-University Giessen, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

Introduction

Currently, world bread wheat production is threatened by wheat stem rust caused by the fungus *Puccinia graminis* f. sp. *tritici* Eriks. & E. Henn (*Pgt*) due to the spread of a new dangerous race designated as Ug99 (Singh et al. 2008). Stem rust was under control in most parts of the world through eradication of common barberry plants in North America (Roelfs et al. 1992) and deployment of resistance genes, such as *Sr31*, *Sr24, Sr2* and *Sr26* in many wheat producing countries (Bariana et al. 2004; Park 2008). The appearance of Ug99 and its variants that are virulent against the above-mentioned genes (Pretorius et al. 2000; Jin et al. 2008) has brought the disease to the forefront as a potential threat to world wheat production. Ug99 was first discovered in Uganda in 1999 (Pretorius et al. 2000) and later spread to other parts of Africa and the Middle East (Singh et al. 2006; Nazari et al. 2009). It is projected to spread farther to the major wheat growing regions of Asia (Singh et al. 2008). Surveys and varietal tests in 18 African and Southeast Asian countries that are lying in the projected route showed that the varieties exhibiting resistance to Ug99 account for only 5% of the total estimated area of 75 million ha wheat fields (Singh et al. 2008). In addition, out of a total of 5,700 common and 2,733 durum wheat

landrace accessions collected from around the world, only 251 (4.4%) and 290 (10.9%), respectively, turned out to be resistant to Ug99 (Bonman et al. 2007). If this race is allowed to spread unchecked, it may have disastrous consequences in world wheat production. All these suggest the urgent need for incorporating effective resistance genes in wheat cultivars cultivated in countries where Ug99 already exists as well as in countries potentially threatened by it.

Experimental tests have shown that there are only few described resistance genes that are effective against Ug99, i.e., *Sr13*, *Sr14*, *Sr24, Sr36* and *SrTmp* (Singh et al. 2008). However, genes, such as *Sr24* and *Sr36* have rendered ineffective against variants of Ug99 that were detected very recently in Kenya (Jin et al. 2008, 2009). *Sr13*, on the other hand, is effective against both variants of Ug99 as well as against a wide range of races (McIntosh et al. 1995; Singh et al. 2006; Admassu et al. 2009). *Sr13*, which was assigned to the long arm of chromosome 6A by cytogenetic methods (McIntosh 1972), was derived from *T. turgidum* var. *dicoccum* cv. Khapli C.I.4013, and was later transferred to the common wheat line, Khapstein (McIntosh et al. 1995).

It is also important to note that some virulent races other than Ug99 are reported to *Sr13* in some countries (Huerta-Espino 1992; McIntosh et al. 1995). Hence, it can be best utilized in combination with other genes through gene pyramiding. The use of race-specific resistance genes in combinations (gene pyramiding) is described as the best strategy in wheat breeding to avoid fast breakdown to stem rust (Singh et al. 2008; Tsilo et al. 2007). For this, molecular markers provide a powerful tool to identify plants that carry combinations of resistance genes. In view of the effectiveness of *Sr13* against Ug99 and other wide ranging races, the identification of molecular markers closely linked to this gene will facilitate an enhanced incorporation of this gene into adapted wheat cultivars by marker-assisted selection procedures. Therefore, the objective of this study was to identify microsatellite markers which are especially suited for marker-assisted selection procedures as they are easy to handle and inherited in a co-dominant manner closely linked to *Sr13*.

Materials and methods

Plant materials

An F_2 mapping population was derived by crossing the susceptible cultivar 'Morocco' with the near-isogenic wheat line Khapstein/9*LMPG that carries the stem rust resistance gene *Sr13*. A total of 158 segregating F_2 individuals were developed and used for marker development. Seeds from each F_2 plant were harvested separately to develop F_3 families.

Phenotyping

Spores of TTKSK (race Ug99) were mixed with sterile water, and inoculated into 7-day-old seedlings of F_2 plants and the parents. Race Ug99 is virulent to cultivar Morocco, but avirulent to Khapstein/9*LMPG. Besides this, 20–25 seedlings of each of the $F_{2:3}$ families were phenotyped with the same pathogen race to distinguish between homozygous and heterozygous resistant F_2 plants, and at the same time to confirm the disease scores on F_2 seedlings.

Immediately after inoculation, seedlings were incubated in the dark for 18 h at 18°C and high relative humidity in a humid chamber. After 18 h of darkness, the seedlings were exposed to fluorescent light for 3 h. Then, they were transferred to a growth chamber and grown constantly at 22 ± 1°C, a light intensity of 10,000 lx and a photoperiod of 16 h. Infection types were scored 14 days after inoculation based on a standard 0–4 scale (Stakman et al. 1962; Roelfs 1988).

DNA extraction and microsatellite analysis

DNA from F_2 progenies and parents was extracted following the method developed by Stein et al. (2001) for wheat. The concentration and quality of DNA were estimated using the NanoDrop ND-1000 spectrophotometer (PeQLab, Erlangen, Germany) and gel electrophoresis.

47 microsatellite markers from the chromosome 6A of wheat (barc1, barc3, barc23, barc37, barc104, barc107, barc113, barc118, barc146, barc171, barc204, barc206, barc1055, barc353, barc1165, cfa2114, cfd2, cfd80, cfd82, cfd190, cfe132, cfe168, cfe179, cfe273, dupw167, gpw5210, gpw7388, gwm132, gwm169, gwm334, gwm417, gwm570, gwm617, wmc145, wmc150, wmc179, wmc182, wmc201, wmc256, wmc398, wmc417, wmc753, wmc786, wmc580, wms427, wms459, wms494) were screened for polymorphism between parents. All polymorphic markers were assayed in all individual F_2 progenies and parents. Each PCR reaction contained 1 µl of 10 × buffer, 1 µl of 25 mM $MgCl_2$, 0.2 µl of each 10 mM dNTPs, forward primer (1.0 pmol/µl), and reverse primer (10.0 pmol/µl), 0.18 µl of 5 U HOT FIREPol® DNA polymerase (Solis BioDyne, Tartu, Estonia), and 1 µl template DNA (25 ng) in a final volume of 10 µl. For SSR amplification, M13 tailed forward primers were used, so that 0.18 µl of 'M13' primer (10.0 pmol/µl) (5′-CAC GAC GTT GTA AAA CGA C-3′) labelled with 5′ fluorescent dyes was added to the reaction mix (Macdonald et al. 2006).

Amplification of DNA was conducted on a GeneAmp® PCR System 9700 (Applied Biosystems). PCR conditions were as follows: 94°C for 5 min; followed by touchdown PCR with 12 cycles of 30 s at 94°C, 30 s at 62°C, 30 s at 72°C; and then 35 cycles with 30 s at 94°C, 30 s at 56°C,

30 s at 72°C, and a final extension at 72°C for 10 min. Amplification products were diluted 1:6 to 1:50 in HPLC gradient grade water (Carl Roth, Karlsruhe, Germany) depending on the intensity of the PCR product. 1 µl of the diluted PCR product was mixed with a mixture of Hi-Di™ formamide (Applied Biosystems) and GeneScan™—500 ROX™ size standard (Applied Biosystems) (0.03 µl ROX:14 µl HiDi™ formamide). The mixture was then denatured at 94°C for 5 min and later subjected to capillary electrophoresis in an ABI PRISM® 3100 genetic analyzer (Applied Biosystems). Data were collected using 3130xl data collection software, v3.0 (Applied Biosystems). The size of the detected alleles was determined using the GeneMapper® software, v4.0 (Applied Biosystems).

Linkage analysis

The F_2 individuals were classified as homozygous resistant (identical allele size to the resistant parent), homozygous susceptible (identical allele size to the susceptible parent) and heterozygous resistant (having both parental allele sizes). Later, the goodness-of-fit to the Mendelian segregation patterns of $F_{2:3}$ (1:2:1) and $F_{3:4}$ (3:1) was tested using Chi-squared (χ^2) distribution analyses. Based on these data, the genetic map was constructed using the software JoinMap, v.4 (Van Ooijen 2006) applying the Kosambi function (Kosambi 1944) and a LOD score 3.

Validation tests

The diagnostic value of the markers linked to *Sr13* was tested across cultivars with diverse genetic backgrounds that were susceptible as well as resistant to Ug99. Four wheat lines known to carry *Sr13*, including the donor of resistance cv. Khapli and 20 wheat cultivars that were tested to be susceptible to Ug99 (Table 2) as well as the susceptible recurrent parent LMPG of the resistant parent Khapstein/9*LMPG and Chinese Spring, were used to determine the diagnostic value of the mapped SSR markers. *Sr13* carrying genotypes were obtained from the University of Minnesota, Dr. Yue Jin, and Agriculture and Agri-Food Canada, Winnipeg, Dr. Thomas Fetch. Respective lines are available from the first author on request.

Results

Phenotypic analysis

The results of the inoculation test showed that cultivar 'Morocco' was highly susceptible (4+ infection type) to TTKSK, whereas the parental line 'Khapstein/9*LMPG' was resistant showing an infection type of 2. The 158 F_2 individuals segregated at a ratio of 121R:37S which fits to a Mendelian 3r:1 s ratio (Table 1). Phenotyping of the F_3 families showed that the F_2 population had a genetic make-up of homozygous resistance (44 plants), heterozygous resistant (77 plants) and homozygous susceptible (37 plants). Chi-square test indicated that this ratio fits to a 1:2:1 segregation ratio (Table 1). These results clearly indicate that resistance to Ug99 in this population is due to a single dominant resistance gene in the resistant parental line. Assay results of the *Sr13* locus and the nine marker loci fitted the 1:2:1 ratio with P values >0.20 (data not shown).

Linkage analysis

The parents, Morocco and Khapstein/9*LMPG, as well as susceptible and resistant bulks were screened with 47 microsatellites which were previously mapped on chromosome 6A. Out of 47 markers, 16 (34%) were polymorphic (15 co-dominant and 1 dominant). The allele sizes of those markers which could be mapped are given in Table 2. All of the polymorphic SSRs were assayed across the mapping population of 158 F_2 progenies. Markers barc118, wmc417, wmc580, cfe179, dupw167 could be integrated into the map only at LOD score 1.0 and were excluded, therefore. Markers cfe168 and gwm334 deviated significantly from the expected 1:2:1 or 3:1 ratio; hence, they were eliminated from the linkage analysis. Nine of the remaining polymorphic markers revealed linkage to *Sr13* (Fig. 1) at LOD score 3. All of the mapped markers were co-dominant. The stem rust resistance gene *Sr13* was mapped 4.0 cM distal to the closest marker, i.e., barc37, and 6.4 cM proximal to wmc256. In the physical map of Sourdille et al. (2004), flanking markers of *Sr13*, barc107 and barc37, are tentatively placed in the combined deletion bin of C-6AL5-0.55 and 6AL4-0.5-0.90, while wmc256 was placed in the 6AL4-0.55-0.90 deletion bin.

Table 1 Segregation ratio and Chi-square test analysis of F_2 and F_3 progenies inoculated with race Ug99

Generation	Resistant	Segregating	Susceptible	Total	Expected ratio	χ^2	P value
F_2	121	–	37	158	3:1	0.211	0.646
F_3	44	77	37	158	1:2:1	0.722	0.697

Table 2 Allele sizes detected on markers linked to *Sr13* on parental lines and a set of resistant and susceptible wheat cultivars

Cultivar	barc146	barc107	barc1165	barc37	*Sr13*[a]	wmc256	gwm570	wmc179	barc353	cfd2
Khapstein/9*LMPG	182[b]	201	159	175	+	134	171	254	243	297/309
Khapstein	182	201	159	175	+	134	171	254	243	297/309
Combination	182	201	159	–	+	134	163/165	254	243	297/309
W2691Sr13	182	201	159	175	+	134	171	254	–	297/309
Khapli	182	201	159	175	+	134	171	254	243	297/309
LMPG	182	201	157	169	−	134	167	268	243/251	295/311
Morocco	180	193	157	181	−	144	163	216	240/254	299/313
Merin	180	193/205	157/159	181	−	144	163	216/254	244/254	299/309/313
Samuno	180	193/205	157	184	−	148	161/163	216	240/254	297/299/313
Vanek	180	201/205	157	181	−	144	161	216/254	240/244	297/299/309
Taifun	180	201/205	157	181	−	144	160/162	216/254	240/244/254	297/309
KWS scirocco	180	205	157/159	184	−	148	165	216/254	240/244	297/299/309
KWSChamsin	180	205	157	181	−	144	163	216/254	240/242	297/299/309
Melisos	180	193/201/205	157	181	−	144	171	216	240/254	297/299/313
Ethos	180	201	157/159	181	−	144	–	216/254	240/244	299/309/313
Naxos	180	205	157	181	−	144	163	216/254	240/244	297/299/309
Thasos	–[b]	201	–	181	−	144	154	–	–	–
PZO59634	180	201	157	181	−	148	163	216	240/254	–
PZO59635	180	193	157	181	−	144	162	216/254	240/244/254	297/309/313
PZO59636	180	193/205	157	181	−	148	156/158/160	216/254	240/244	297/299/309
PZO59638	180	205	157/159	181	−	144	160	216/254	240/244/254	297/309/313
PZO59640	180	205	157	184	−	144	–	216/254	240/242/254	297/299/309/313
PZO59641	–	201/205	–	181	−	148	163	–	–	–
PZO59642	–	193/205	–	181	−	144	163	–	–	–
Kubsa	180	193	157/159	181	−	144	171	216	240/244/254	299/309/313
Galama	180	193	157	181	−	144	163	216/254	240/244/254	297/299/309/313
Chinese spring	172	205	159	178	−	134	163	218	243/245	297/311

–: Null allele or failed PCR

[a] +: *Sr13* present, −: *Sr13* absent

[b] Size of amplified fragments in base pairs (bp)

Validation test

Out of the linked markers tested only barc37 amplified a fragment that turned out to be diagnostic for the stem rust resistance gene *Sr13* in our set of genotypes analysed. A band size of 175 base pairs (bp) amplified by the SSR marker barc37 was detected in those lines carrying *Sr13*, while in the other wheat cultivars that were susceptible to Ug99, indicating the absence of *Sr13*, fragments of different size were detected (Table 2). All carriers of *Sr13* showed the same fragments for all markers as the donor of *Sr13*, i.e., cv. Khapli.

Discussion

Sr13 is an important source of resistance to stem rust in the face of Ug99. In addition to its proven effectiveness against Ug99, virulence for *Sr13* appears to be extremely rare except in a few countries (Luig 1983; Huerta-Espino 1992; Admassu et al. 2009). Because of this, it has already been exploited in some Australian wheat cultivars as a source of resistance, but cultivars carrying this gene, in general, have low 1,000 grain weight (McIntosh et al. 1995). This may be a reason for the underutilization of *Sr13* despite its potential for stem rust control. Therefore, long lasting backcrossing procedures are needed to combine *Sr13* with superior agronomic performance. This can be considerably enhanced using marker-assisted selection procedures (e.g. Ordon et al. 2003; Dong et al. 2007).

In the present study, such markers were developed and *Sr13* was assigned to chromosome 6AL of wheat. This map location is consistent with previous mapping studies based on the cytogenetic techniques positioning *Sr13* on chromosome 6AL of wheat (McIntosh 1972; Klindworth et al. 2007). Chromosome 6A carries an additional stem

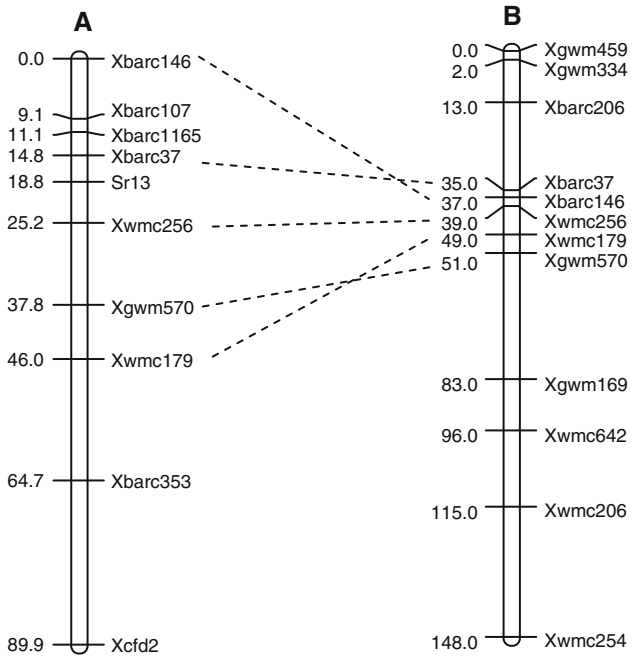

Fig. 1 Genetic linkage map of *Sr13* compared to the consensus map of chromosome 6A. **a** Genetic linkage map of *Sr13* constructed using simple sequence repeat (SSR) markers from the long arm of chromosome 6A. The map was constructed from 158 F_2 individuals derived from a cross between Khapstein/9*LMPG and Morocco. **b** Consensus map of chromosome 6A modified after Somers et al. (2004)

rust resistance genes *Sr8* and *Sr26* (McIntosh 1972; McIntosh et al. 1995; Mago et al. 2005). Another gene designated as *Srdp2*, which was initially thought to be allelic to *Sr13* (McIntosh et al. 1995), but later confirmed as a different gene (Huerta-Espino 1992) was also located on this chromosome.

The order of markers obtained in our study is in agreement with those previously published (Somers et al. 2004; Zhang et al. 2008). The order of three markers out of five corresponds to the consensus map of Somers et al. (2004). According to the latter authors, barc37 is located proximal to barc146, however, the present study located barc37 distal to barc146 as shown in Fig. 1. Such discrepancy in marker order on a genetic map which was also detected for gwm570 and wmc179 is not uncommon as witnessed by Tsilo et al. (2007) and Song et al. (2005). The deviation of co-linearity in marker order between the two maps could be explained by the fact that the map of Somers et al. (2004) was a consensus map constructed by joining four independent genetic maps of wheat. On the other hand, the order of markers, barc146, barc107, wmc256, gwm570, wmc179, barc353 and cfd2, is in complete agreement with the consensus map of Zhang et al. (2008). barc37 and barc1165 were not present in this map, and hence, its position could not be compared to our result or to that of Somers et al. (2004). Overall, our marker order is in agreement with previously published maps. According to Sourdille et al. (2004), the two flanking markers proximal to *Sr13*, barc107 and barc37, were tentatively placed in the combined bin of C-6AL5-0.55 and 6AL4-0.5-0.90 on chromosome 6A of Sourdille's physical map. Only the marker mapped distal to *Sr13*, wmc256, was placed in the 6AL4-0.55-0.90 bin. Therefore, the bin location of *Sr13* is not conclusive.

The SSR marker barc37 that is closely linked to *Sr13* produced a fragment that in our set of genotypes analysed is diagnostic for *Sr13*. On the other hand, the other markers tested produced mixed allele sizes both in the resistant as well as in the susceptible lines and cultivars, indicating that they do not have a diagnostic value. Hence, SSR marker barc37 is a good candidate for marker-assisted selection. To some extent, this holds true also for wmc256. Respective markers have also been developed for other stem rust resistance genes (Mago et al. 2005; Tsilo et al. 2007; Bansal et al. 2008). A combined use of these markers may lead to a reduction in the selection of false positives, but may also lead to a larger linkage drag (Zeven et al. 1983; Hospital 2001).

Even though there is virulence to *Sr13* in some countries, its effectiveness against Ug99 makes it a highly valuable source of resistance in the present day breeding programmes. But using rust bioassays while stacking two or more resistance genes is a time-consuming practice, and sometimes it is difficult to determine the presence of all resistance genes in the new cultivar unambiguously, especially for stem rust resistance genes that are broadly effective (Mago et al. 2005; Tsilo et al. 2007). PCR-based markers help to identify and stack these genes. Therefore, these markers will be useful for marker-assisted integration of *Sr13* into newly developing elite wheat breeding lines or increase the efficiency of incorporating *Sr13* to commercial cultivars that are widely adopted, but susceptible to Ug99, thereby help to achieve durable stem rust control in wheat.

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