DETECTION OF RAPD MARKERS FLANKING THE LEAF RUST RESISTANCE GENE, *Lr* 34, IN WHEAT, USING BULK SEGREGANT ANALYSIS

M.N Barakat [1], M.I.Motawei [1], S.I Milad [1], H.M. Abouzied [2] and A.A. Abou-Ali[3].

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ABSTRACT

Leaf rust, caused by Puccinia triticina, is an important disease of bread wheat (Triticum aestivum L.) in several production areas of the world .The most effective and economical approach for controlling leaf rust disease is to use resistant cultivars. The present objectives were to map the resistance gene, Lr34, in the breeding materials and develop RAPD- (PCR) based markers for marker-assisted selection (MAS). RAPD-PCR (polymerase chain reaction) analysis was conducted, using bulked segregant analysis (BSA) in a mapping population of sixty F_2 individuals derived from a cross between the susceptible cultivar, Sakha 69, and the resistant monogenic line, Lr34. After performing RAPD-PCR analysis with twenty arbitrary 10-mer primers and agarose-gel electrophoresis, mapping of two markers flanking Lr34 was reported. The closest marker was Pr_2 (5'AGGTACCGG3') at the 2.8 cM with logarithm of the odds to the base 10 (LOD) scores of 15.1, and the other one Pr_1(5'GACCGCTTGT3') was at 16.3 cM with LOD scores of 6.5. Bulked segregant analysis with RAPD and linkage mapping might facilitate selection and enable gene pyramiding for leaf rust resistance in wheat breeding programs.

Key words: *MAS, marker-assisted selection, PCR, polymerase chain reaction, leaf rust, Lr34, bulked segregant analysis (BSA), random amplified polymorphic DNA (RAPD).*

INTRODUCTION

Wheat is the most important winter crop in Egypt. Leaf rust, caused by *Puccinia triticina*, is one of the most important diseases of wheat in Egypt. The main problem facing the Egyptian wheat cultivars is the appearance of new leaf rust races that causes many new cultivars, including high yielding ones, to be eliminated. Egypt, also, is facing many challenges due to the increasing population density, in which the annual birth growth rate has reached 2.1%, in addition to the shortage of arable land. The main target of the agricultural policy , in Egypt, is to increase the wheat production, in specific, as well as the other food crops, in general, to decrease the gap between wheat production (50-55%) and the annual requirements (about 12 million tons).

Breeding for durable resistance against the fungal leaf rust disease in wheat is based on the combination of different leaf rust (*Lr*) resistance genes in one cultivar. More than 57 different leaf rust (*Lr*) races have been catalogued (McIntosh *et al.*, 2005). The selection of genotypes, containing several leaf rust resistance genes, using infection with leaf rust isolates with defined avirulence genes, is very time consuming. The development of molecular markers for specific *Lr* genes allows the detection of these genes independently of the phenotype.

Bulk 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 (Michelmore *et al*, 1991). BSA has been successfully used to develop molecular markers for wheat leaf resistance (Sybil *et al*, 2007). Genotyping of other wheat fungal diseases, using bulk segregant analysis, has been reported (Qing *et al.*, 2005and Weihua *et al.*, 2005).

The development of molecular markers linked to a resistance gene represents a useful tool for plant breeding, as the presence of the gene could be detected without waiting for the phenotypic expression of this gene. Moreover, it allows for simultaneous screening of several disease resistance genes. In wheat, leaf rust resistance genes have been molecularly tagged, with RAPD marker (Xiao *et al.*, 2004; Sudhir *et al.*, 2005 and 2006). In the present study, molecular markers were developed, based on RAPD and PCR technologies for the leaf resistance gene, *Lr*34. These markers could be valuable to combine *Lr*34 with other effective resistance genes to improve the durability of leaf rust resistance.

MATERIALS AND METHODS

Plant material:

Identification of RAPD markers, linked to *Lr*34 gene, were carried out on mapping population of sixty F_2 individuals, derived from a cross between the resistant monogenic line, *Lr*34, and the susceptible cultivar, Sakha 69. The cross was made during the season of 2003/2004 and F_1 was selfed in2004/2005 to produce the mapping population of F_2 in 2005/2006.

Leaf rust resistance evaluation:

Resistance to *Puccinia triticina* was tested in 2005/2006 season at the adult stage at the Experimental Farm of Field Crop Research Institute, Sakha Agriculture Research Station, using a mixture of

1. Biotechnology Laboratory, Crop Science Department, Faculty of Agriculture, Alexandria University, Egypt.
2. Crop Science Department, Faculty of Agriculture (Damanhour Branch), Alexandria University, Egypt.
3. Plant Pathology Department, Agricultural research Station, ARC, Egypt.

the common races under the Egyptian conditions. Disease reaction was recorded at 12-14 days after inoculation, using the 0-4 scale (Stakman *et al.*, 1962). After twenty days from inoculation, green leaves from young F_2 plants were collected for RAPD analysis.

DNA extraction:

Genomic DNA was extracted from fresh leaves of the monogenic lines, Sakha 69 and individual F_2 plants of each cross, using CTAB (Saghai - Maroof *et al.*, 1984). RNA was removed from the DNA preparation by adding 10µl of RNAase (10mg /ml) and, then, incubated for 30 min. at 37°C. Sample DNA concentration was quantified by using a spectrophotometer (Beckman Du-65).

PCR amplification:

Twenty RAPD primers, previously tested by Barakat *et al* (2001), were used in the present experiment to amplify the template DNA. Amplification reaction volumes were 25 µl, each containing 1 x PCR buffer with $MgCl_2$ (50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2 mM $MgCl_2$ and 1% trition x-100), 200 µM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 U of tag polymerase. Reaction mixtures were exposed to the following conditions: 94°C for 3 min. followed by 45 cycles of 1 min. at 94°C, 1 min. at 36°C, 2 min. at 72°C, and a final 7 min. extension at 72°C. Amplification products were vizualized with DNA marker on 1.6% agarose gel with 1x TBE buffer and detected by staining with an ethidium bromide solution for 30 min. Gels were, then, destained in deionized water for 10 min. and photographed on Polaroid films under UV light.

Bulked segregant analysis of the leaf rust resistance gene, *Lr* 34 :

Four different bulks were created for both phenotypic classes of plants, as follows: bulks 1 R (resistant) and 1S (susceptible), respectively, a mix of equal amounts of DNA from five resistant and five susceptible were chosen at random. Each of the RAPD primers was simultaneously screened on these four DNA bulks and on the parental cultivars, monogenic cultivar and Sakha69. Based on the evaluations of DNA bulks, sixty individual F_2 plants were analyzed with co-segregating primers to confirm RAPD marker linkage to the *Lr*34 gene.

Data analysis:

Goodness of fit to a 9:7 ratio was calculated for RAPD marker by Chi-square test. A regression analysis was performed between the RAPD marker and the values of leaf rust resistance gene of the F_2 lines (Morens & Gonzales, 1992).

Linkage analysis:

Map manager QTX Version 0.22 (Meer *et al.*, 2002) was used to analyze the linkage relationship of RAPD markers detected from bulked segregant analysis. Linkage was detected when a log of the likelihood ratio (*LOD*) threshold of 3.0 and maximum distance was 50 *cM*. The Kosambi's mapping function was used.

RESULTS AND DISCUSSION

RAPD markers linked to *Lr* 34 :

The F_2 mapping population, derived from the cross between the resistant *Lr34* donor line (monogenic line) and the susceptible cultivar, Sakha 69, was used. The two parents and the F_2 generation were screened with twenty RAPD primers (Barakat *et al.*, 2001). Two of these primers, *Pr1*(5'GACCGCTTGT3') and Pr_2 (5'AGGTGACCGG3') generated polymorphic DNA fragments linked to *Lr*34 gene. These two markers were present in the resistant bulk F_2 lines and in the resistant monogenic parent *Lr*34 (Fig.1). The 400bp fragment, amplified by Pr_1,was present in the monogenic line (resistant parent),but absent in the susceptible parent, Sakha 69.This marker (Pr_1), also, was present in the resistant bulked DNA, but was not in the susceptible bulked DNA (Fig.2). The 350bp fragment, amplified by Pr_2,was present in the monogenic line (resistant parent),but absent in the susceptible parent, Sakha 69. This marker (Pr_2), also, was present in the resistant bulked DNA, but was not in the susceptible bulked DNA (Fig.3). The two markers, primer (Pr_1) and primer(Pr_2), were further used to check its linkage with the leaf rust resistance gene (*Lr*34), using mapping of F_2 population, derived from the cross between the resistant *Lr*34 donor line (monogenic line) and Sakha 69. In the RAPD marker primer (Pr_1), 25 out of the sixty individuals, in the population, exhibited the amplified polymorphic fragment (400bp), while, the remaining 35 did not. The ratio fitted the expected Mendelian ratio, 7:9 (x^2= 0.11, $p< 0.01$). In the RAPD marker primer (Pr_2), 24 out of the sixty individuals, in the population, exhibited the amplified polymorphic fragment (350bp), while, the remaining 36 did not. The ratio fitted the expected Mendelian ratio, 7:9 (x^2= 0.34, $p< 0.01$).

A regression analysis was performed to test the significance of the linkage between *Lr34* and the polymorphic markers. The results showed that the regression analysis for the Pr_1 and Pr_2 were significant. The calculated r^2 for Pr_1 and Pr_2 were 0.32 and 0.68, respectively. This indicated that the two markers were linked with the leaf rust resistant gene, *Lr*34.

Several molecular markers, such as restriction fragment length polymorphisms (RFLPs), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs), cleaved amplified polymorphic sequence (CAPS), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs), intersimple sequence repeat (ISSR), diversity arrays technology (DArT), and single nucleotide polymorphism (SNP) have been used to construct linkage map in several plants. Each marker system has advantages and disadvantages, and the various factors to be considered in selecting one or more of these marker systems have been reviewed by Semagn *et al*, (2006). Random amplified polymorphic DNA (RAPD; Williams *et al*, 1990) was embraced in different laboratories, especially those in the developing countries, due to its low cost, compared to other DNA-based techniques, such as amplified fragment length polymorphism or AFLP (Vos *et al.*, 1995) and simple sequence repeats or SSRs (Bruford and Wayne, 1993). Besides, RAPD protocol is fairly simple, while protocols like AFLP and SSR are technically demanding (Karp *et al.*, 1997). RAPD markers have been utilized in identifying and constructing genetic linkage mapping for the detection of different leaf rust (*Lr*) genes in wheat (Dedryver *et al* ,1996; Barakat *et al*, 2001; Khan,*et al*, 2005; Cherukuri *et al*, 2005; Sudhir *et al*, 2006).

Bulked segregant analysis (BSA) is, generally, used to tag genes controlling simple and quantitative traits (Wang and Paterson, 1994). High- throughput or high-volume marker techniques, such as RAPD or AFLP that can generate multiple markers from a single DNA preparation, are, generally, preferred for BSA (Qing *et al* 2005; Weihua *et al*, 2005and Sybil *et al*, 2007).

Mapmanager analysis:

After performing mapmaker linkage analysis on the mapping population of the sixty F_2 individuals, two primers (Pr_1 and Pr_2) out of the twenty RAPD primers were shown to be linked to *Lr*34 gene and to be flanked *Lr*34 within a distance of about 19.1 *cM* (Fig. 4). A standard maximum-likelihood technique was employed to analyze the linkage between *Lr*34 and the two linked marker loci. The map distance between *Lr*34 gene and Pr_1 was 16.3 *cM* and between *Lr*34 gene and Pr_2 was 2.8 *cM* with *LOD* scores of 6.5 and 15.1, respectively. *LOD* values of more 3 were typically used to construct linkage maps (Risch, 1992). The distance between the RAPD marker, produced by the primers Pr_2 and the *Lr*34 gene, was less than 10 *cM*, so it was considered to be tightly linked to the *Lr*34 gene. A number of several molecular markers flanking different genes of leaf rust, at different distances, have been reported. Sudhir *et al* (2006) generated a saturated region carrying 25 molecular markers linked to the gene, *Lr19*, within 10.2 *cM* on either side of the locus. Genetic linkage between RFLP marker(*csLV34*) and *Lr34/Yr18* was estimated at 0.4 *cM* by Lagudah *et al.*, (2006). A coupling phase, linked RAPD marker $S464_{721}$, and a repulsion phase, linked RAPD marker $S326_{550}$, flanked the gene *Lr28* by a distance of 2.4 *cM* on either side,as reported by Cherukuri *et al* (2005). Khan et *al* (2005) found that ISSR marker UBC 840_{540} to be linked with *Lr3a* in repulsion at a distance of 6.0 *cM*. Markers cfa2019 and cfa2123 flanked stem rust *Sr22* at a distance of 5.9 *cM* (distal) and 6.0 *cM* (proximal), respectively. Xing *et al* (2007) obtained seven markers linked to *Lr19* resistant gene, ranged from 3.3 *cM* up to 9.6 *cM*, all of these of the seven specific fragments were isolated from the polyacrylamide gels, reamplified, cloned and sequenced. The investigators suggested that their result might facilitate genetic mapping, physical mapping and the eventual cloning of *Lr19*.

One of the main uses of DNA markers in agricultural research has been in the construction of linkage maps for diverse crop species. Linkage maps have been utilized for identifying chromosomal regions that contain genes controlling simple traits (controlled by single gene) and quantitative traits using QTL analysis (Sun *et al*,1997). The present study indicated that RAPD markers, combined with bulk segregant analysis, could be used to identify molecular markers linked to leaf rust resistance gene in wheat. Once these markers are identified, they could be used as marker-assisted selection in early generation of the breeding programme.

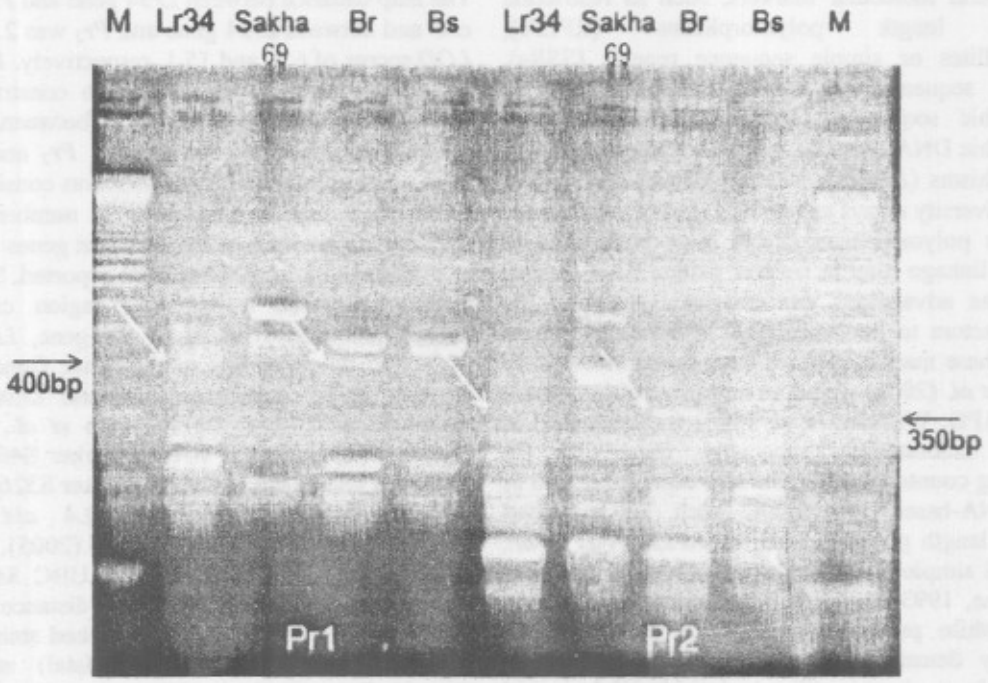

Fig. 1: RAPD fragments produced by primers Pr_1 and Pr_2 M: Molecular weight , followed by P1 and P2 are parents , Monogenic and Sakha 69, resp., Br, bulk resistance; Bs bulk susceptible F_2 individuals in Monogenic X Sakha 69 cross (R: resistant ; S: susceptible). Arrows indicate the position of the specific bands.

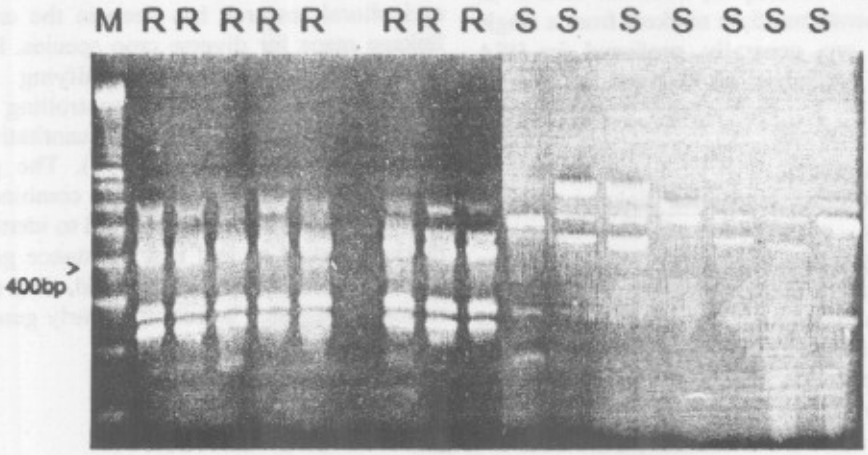

Fig .2 : RAPD fragments produced by primer Pr_1, M: Molecular weight , followed by F_2 individuals in Monogenic X Sakha 69 cross (R: resistant ; S: susceptible). Arrows indicate the position of the specific bands.

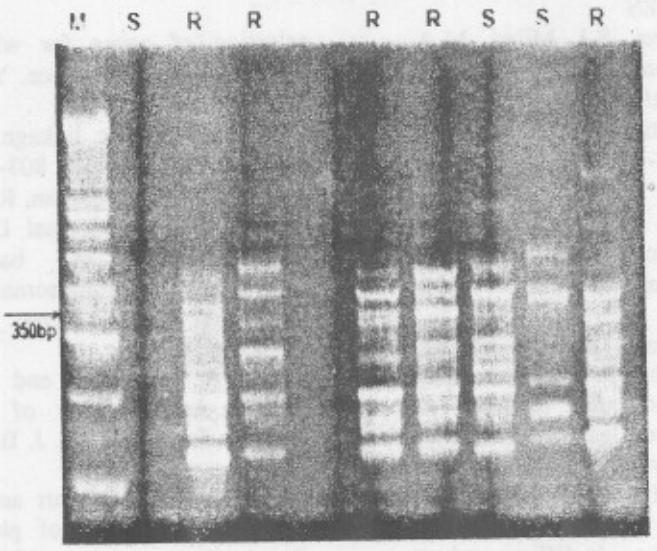

Fig. 3 : RAPD fragments produced by primer Pr_2, M: Molecular weight , followed by F_2 individuals in Monogenic X Sakha 69 cross (R: resistant ; S: susceptible). Arrows indicate the position of the specific bands.

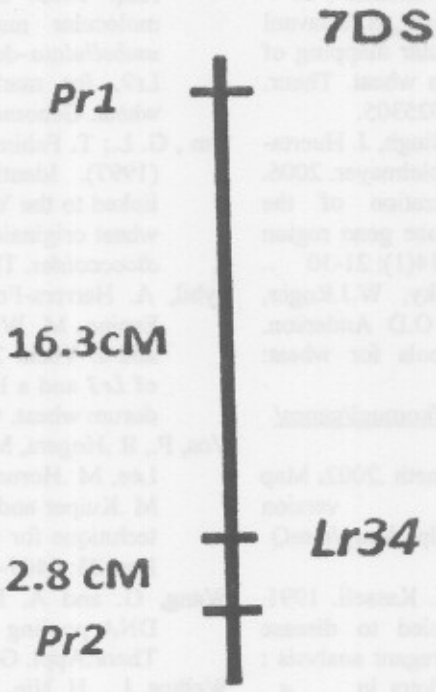

Fig.4: Linkage map showing the two markers flanking *Lr*34 .All distances are given in centiMorgan using Kosambi's mapping function.

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الملخص العربى

الكشف عن دلائل RAPD المطوقة لجين المقاوم لصدأ الأوراق *Lr* 34 فى القمح باستخدام تحليل BSA (تحليل الانعزالات بطريقة التجميع)

محمد نجيب بركات[١] ، محمد إبراهيم مطاوع[١]، سناء ابراهيم ميلاد[١] هناء مهدى ابوزيد[٢]
عبد العزيز ابو على[٣]

معمل التقنية الحيوية- قسم المحاصيل – كلية الزراعة – جامعة الاسكندرية- مصر
قسم المحاصيل – كلية الزراعة بدمنهور – جامعة الاسكندرية- مصر
قسم امراض النبات محطة البحوث الزراعية (سخا) – مصر

يعتبر مرض صدأ الأوراق المتسبب بواسطة *Puccinia triticina* مرض مهم في قمح الخبز (*Triticum aestivum* L.) في كثير من مناطق الإنتاج في العالم. يعتبر إستخدام الأصناف المقاومة أهم العوامل المؤثرة في التحكم في هذا المرض.

الهدف من هذه الدراسة هو تحديد موقع الجين المسئول عن المقاومة لصدأ الأوراق*Lr*34 في مواد التربية. وتطوير دلائل RAPD- (PCR) المرتكزة على الإنتخاب المبني على أساس الدلائل الجزئية (MAS) . تحليل RAPD-PCR تم اجراؤه بأستخدام طريقة Bulked segregant analysis (BSA) في عشيرة من ستين نبات فردي للجيل الثاني الناتج من التهجين بين صنف القمح "سخا -٦٩" القابل للاصابة بصدأ الأوراق وسلالة المقاومة "Monogenic" المحتوية على الجين المقاوم (*Lr*34) . وبعد استخدام عشرين بادىء مع تحليل RAPD-PCR وباستخدام الهجرة الكهربية تم تحديد موقع دليلين مطوقين لجين *Lr*34 وكان الأقرب هو(5'AGGTACCGG3') *Pr*2 مسافة ٢.٨ سنتيمورجان (LOD=15.1) والأخر (*Pr*1(5'GACCGCTTGT3' وجد على مسافة ١٦.٣ سنتيمورجان (LOD=6.5) . ويمكن استخدام تحليل BSA مع RAPD معتمداً على الدلائل الجزيئية في تحديد عدد من الجينات الوراثية المسئولة عن المقاومة لصدأ الأوراق القمح وبالتالي تحسين برامج التربية في القمح للمقاومة لصدأ الأوراق .