IDENTIFICATION OF MOLECULAR MARKERS LINKED TO STRIPE RUST RESISTANCE REGION LR34/YR18 IN WHEAT

M N. Barakat[1], H. M. Shakam[2], M. I. Motawei[1], M.A.EL-Sahrigy[2] and A.M. Abou-Aly[3].

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ABSTRACT

RAPD and STS markers were used for tagging and mapping the important slow stripe rust genes, Lr34/Yr18, in F_3 wheat population. F_3 population has been derived from the cross between the stripe rust resistant parent (monogenic Lr34 line) and the susceptible parent (Sids1) and the evaluation of stripe rust was made at seedling stage. Bulk segregant analysis was used in conjunction with RAPD analysis. After mapmaker linkage analysis on the F_3 population, a standard maximum likelihood technique was employed to analyze the linkage between Lr34 and the linked marker loci. The genetic distance between Lr34/Yr18 and the RAPD marker OPB13 (700bP) was 31.2 cM with LOD scores of 2.3. EST- based STS marker $BE493812_{(250bp)}$ was mapped to Lr34/Yr18 gene by using the bulked segregant analysis (BSA) procedures with the same previous population. After mapmaker linkage analysis on the F_3 population, a standard maximum likelihood technique was employed to analyze the linkage between Lr34 and the linked marker loci. The genetic distance between Lr34/Yr18 and STS marker $BE493812_{(250bp)}$ was 28.8 cM with LOD scores of 2.2. RAPD and STS markers revealed that Sids1 did not have Yr18, when evaluated to stripe rust resistance, and did not have Lr 34, when evaluated to leaf rust resistance. In other words, Sids 1 did not have Lr34/Yr18 at both seedling and adult stages. Moreover, identification and mapping of RAPD and STS markers, linked to Lr34/Yr18, in the present study, showed that Lr34/Yr18 was involved in both seedling and adult plant resistance. The availability of such RAPD and STS markers should accelerate the introgression of the resistance gene into current wheat cultivars. It might, also, facilitate the accumulation of several resistance genes in a target cultivar. However, further efforts are needed to narrow down the marked interval spanning the target gene (Lr34/Yr18).

INTRODUCTION

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 consumption (about 12 million tons). In Egypt, wheat rust diseases are still among the main factors decreasing the longevity of the Egyptian wheat cultivars.

Breeding for genetic resistance to rust is the most efficient, cost effective and ecofriendly approach to overcome the losses caused by rust epidemics. However, due to selection pressure and evolution, new and virulent races of the fungus appeared, which increased the need to develop durable resistance. This could be achieved by monitoring slow rusting genes, such as Lr34 and Lr46, which are widely used in the CIMMYT breeding program. These genes have been identified to be race-non specific and could cause durable resistance. Recently, William *et al.*(2006) reported that characterization of slow-rust genes for leaf and stripe rusts, in improved wheat germplasm would enable wheat breeders to combine these additional loci with known slow-rusting loci to generate wheat cultivars with higher levels of slow-rusting resistance.

Molecular markers are powerful tools for identifying quantitative traits and dissecting these complex traits into Mendelian manner in the form of quantitative trait loci (QTL), as well as for establishing the genomic locations of such loci. Bulked segregant analysis (BSA), which involves pooling of entries at the two extremes for a segregating trait (Michelmore *et al.*, 1991), has been effectively used for identifying molecular markers associated with disease resistance genes in a number of species (Reiter *et al.*, 1992; William *et al.*, 2006). BSA and linkage mapping in wheat has, also, enabled identification of molecular markers associated with genes that condition resistance to leaf rust (William *et al.*, 1997; Barakat *et al.*, 2001) and stripe rust (Chague` *et al.*, 1999; Motawei *et al.*, 2003). The present paper described the use of RAPD and STS markers for tagging and mapping of the important slow rusting genes, Lr34/Yr18, in F_3 wheat population.

MATERIALS AND METHODS

Plant material:

Identification of RAPD and STS markers, linked to the slow rusting genes, Lr34/Yr18, were carried out on segregating F_3 population, derived from a cross between the resistant monogenic line, Lr34/Yr18, and the susceptible cultivar, Sids1. The cross was made during the season of 2002/2003 and was selfed in 2003/2004 to produce the F_2 population. Unfortunately, in 2004/2005 season, both artificial and natural infection against stripe rust was not achieved. Hence, the F_3 seeds were obtained for the cross.

1. Biotechnology Laboratory, Crop Science Department, Faculty of Agriculture(El-Shatby) Alexandria University, Alexandria, Egypt.
2. Department of Genetics, Faculty of Agriculture(El-Shatby), Alexandria, Egypt.
3. Plant Pathology Department, Sakha Agricultural Research Station, ARC, Egypt.

Stripe rust resistance evaluation:

Seedlings of the parents and F_3 plants (7-10 days old, grown in pots) were dusted with a mixture, containing uredospores of each rust sample plus talcum powder at the rate of 1:20(w:w), as suggested by Tervat and Cassel (1956). Pots were kept for 48 hrs at 9° C, followed by incubation for 18-20 days at 15-18° C, as adopted by Stubbs (1988), then, the infection types of stripe rust were estimated using the scale of McNeal *et al.* (1971). According to this scale, plants with the infection types, ranging from o to 6 were considered as resistant infection types. While, infection types, ranging from 7 to 9 , were considered as susceptible ones. This evaluation was carried out in a greenhouse at Plant Pathology Department, Sakha Agricultural Research Station, ARS. Egypt.

DNA extraction:

Frozen young leaves (500 mg) were ground to a powder in a mortar with liquid nitrogen. The powder was poured into tubes, containing 9.0 ml of warm (65°C) CTAB extraction buffer (Sagahi-Maroof *et al.*, 1984). The tubes were incubated at 65°C for 60-90 min. and 4.5 ml chloroform/ octanol (24: 1) was added and tubes were rocked to mix for 10 min., and centrifuged for 10 min. at 3200 rpm. The supernatants were pipetted into new tubes and 6 ml isopropanol was added. After 60 min., the tubes were centrifuged for 10 min. and the obtained pellets were put in steriled Eppendorf tubes, containing 400 µl of TE buffer of a pH 8.0 (10 mM Tris-HCl, pH 8.0 + 1.0 mM EDTA, pH 8.0) .The DNA's, from different genotypes, were, then, extracted and stored at -20C until use.

RAPD analysis and PCR amplification:

Nine primers, previously tested by several investigators (Sun *et al*, 1997; Barakat and Imbaby, 2005), from Pharmacia Biotech. (Amersham Pharmacia Biotech UK Limited, England HP79 NA), were tested in the present investigation to amplify the templated DNA.

Amplification reaction volumes were 25 µl, each containing 1 x PCR buffer with MgCl2 (50 mM KCl, 10 mM Tris-HCl (pH = 9.0), 2 mM MgCl2 and 1% trition x-100), 200 µM each of dATP, dCTP, dGTP and dTTP, 50 PM primer, 50 P.g template DNA and 1.5Uof taq 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 2.3% agarose gel with 0.5 X TBE buffer and staining with an ethidium bromide solution for 30 min. Gels were, then, destained in deionized water for 10 min. and photographed on Polarid films under UV light.

STS analysis and PCR amplification:

Two pairs of EST-based STS primers were used to amplify the DNA template in the present investigation. The sequence of the primer BF473324 was L,5' AGCCAACAGTCATTGCAACA 3'; R, 5' AGCCATAGCATCAACAGGCT 3' and the sequence of the primer BE493812 was L,5' TCATATGCCTCGAGAGGGTC 3'; R, 5' ATT GTT ACC CGC CCT TTA CC 3'. Polymerase chain reaction (PCR) amplifications were performed in 25µl reaction with 2.5µl of 10 x magnesium- free PCR buffer, 1.5 µl of magnesium chloride (25 mM), 2.0 µl of dNTPs (2.5 mM each d NTP), different concentrations of the primer, ranging from 0.2µM to 1µM, were used (for each forward and reverse primer) for Lr34, and 100 ng of DNA. Different PCR programs were used. All PCR products were resolved in 3.4 % high-resolution agarose gel in 0.5 x TBE buffer. Amplification products were visualized, as described for RAPD analysis.

Bulked segregant analysis:

Bulked –segregant analysis (Michelmore *et al.*, 1991) was used to target the genomic regions, associated with the stripe rust resistance QTLs. Two bulk DNA samples were constructed, using equal amounts of DNA from five susceptible and five resistant plants selected, based on phenotypic assessments. RAPD and STS primer combinations were, then, screened on the parents and the two bulk DNA samples, from which some primer combinations revealed bands that were polymorophic, not only between parental genotypes, but also between the pair of the bulk DNA. Based on the evaluations of DNA bulks, individual F_3plants were analyzed with cosegregating primers to confirm RAPD and STS markers linkage to the Lr34/Yr18 genes.

Data analysis:

Goodness of fit to the obtained ratios was calculated for RAPD and STS markers by Chi-square test. A regression analysis was performed between the RAPD and STS marker and the values of stripe rust resistance genes of the F_3 plants (Moreno - Gonzales, 1992).

Linkage analysis:

Map manager QTX, Version 0.22 (Meer *et al.*, 2002), was used to analyze the linkage relationship of RAPD and STS 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:

In an attempt to facilitate the pyramiding of adult plant resistance genes in wheat, using molecular markers, it was necessary to identify the markers, which were linked to stripe rust resistance genes and must be simple and accurate to be used. Lr34 and Yr18 genes confer slow rusting resistance to leaf and stripe rusts, respectively, and are known to be pleiotropic or completely linked to each other (McIntoch, 1992; Singh, 1992). Leaf tip necrosis, a morphological trait, showed complete linkage or pleiotropism with Lr34 and Yr18 (Singh, 1992) and could be used in some environments as a marker to identify wheat lines carrying these genes. To identify the RAPD markers, linked to Yr18, the cross between the resistant Yr18 donor line (monogenic Lr34/Yr18 line) and Sids1cultivar was made as the source of the segregating population to identify the RAPD markers linked to the stripe rust resistance genes. The monogenic line possessed the Yr18 gene and was resistant to stripe rust under artificial infection, while, Sids 1 was susceptible under artificial infection. In the F_3 population, bulked DNA from the F_3 individuals, differing in resistance to stripe rust, were used as template for amplification with each arbitray 10-mer oligonucleotide primers. A total of nine primers, previously tested by several investigators (Sun *et al.*, 1997; Motawei *et al.*, 2003; Barakat and Imbaby, 2005) for screening stripe rust resistance genes,were used to identify Yr18 in the donor parent and F_3 population. Primers, that gave clear, distinguishable and reproducible patterns, were considered for analysis.

The 700 bp fragment, amplified by OPB 13, was present in the monogenic line (resistant parent) and absent in the susceptible parent, Sids 1. This marker (OPB 13) was, also, present in the resistant bulked DNA, but not in the susceptible bulked DNA (Fig.1). Interestingly, marker primer OPB 13 (700 bp), which was linked to Yr18 in the monogenic line, Lr34/Yr18 X Sids1 cross, was the same primer at 800 bp fragment, previously shown to be linked to Yr15 (Barakat and Imbaby, 2005).

The RAPD marker (primer OPB 13) was further used to check its linkage to the stripe rust resistance gene, Yr18, using segregating F_3 population, derived from the cross between the resistant Yr18 donor line (monogenic line) and Sids1.For the RAPD marker primer OPB 13, 61 of the eighty individuals in the population ,exhibited the amplified polymorphic fragment (700 bp), while, the remaining did not. The ratio fitted the expected Mendelian ratio, 3:1 (X^2 = 0.067, P < 0.01).

A regression analysis was performed to test the significance of the linkage between Yr18 and the polymorphic markers. The result showed that the regression analysis for the OPB 13 marker was significant. The calculated R^2 for OPB 13 was 69%. This means that the relative contribution to the overall phenotypic variation among F3 plants due to the difference between alternative alleles at RAPD marker (OPB 13 700bp) locus was 69%. Using bulk segregates, in conjunction with RAPD analysis, polymorphic fragment was found to be perfectly linked to one of the wheat yellow rust resistance gene in a large segregating population Chen *et al.*(1998) used bulk segregant analysis to developed RAPD markers for wheat resistance to yellow rust. Identification of RAPD markers linked to stripe rust resistance genes in F2 population of wheat by using the bulked segregant analysis procedure with RAPD markers have been reported. Recently, identification of RAPD markers for the detection of yellow rust resistance gene Yr15 in wheat has been reported (Barakat and Imbaby, 2005).

After Mapmaker linkage analysis on the F3 population, the genetic distance between RAPD marker (OBP13) and stripe rust resistance gene Yr18 was determined to be 31.2 cM with 2.3 LOD (Fig.4). Therefore, RAPD marker (OPB 13) was linked to the quantitative trait loci (QTL) for stripe rust resistance gene Yr18. Similar results were reported using OPB 13 primer for identifying molecular markers for linkage with Yr15 gene (Sun *et al.*, 1997; Barakat and Imbaby, 2005). Sun *et al.* (1997) reported that a 2.8 Kb fragment, produced by the Nor RFLP probe and 1420 bp PCR product generated by the RAPD OPB 13 primer showed linkage, in coupling, with Yr15 gene. The map distances between OPB 13-Yr 15-Nor were 27.1 cM and 11.0 cM for the first and second intervals, respectively. Also, Barakat and Imbaby (2005) reported that the map distance between Yr15 gene and OPB 13 and UBC 321 primers were 7.7 and 13.2 cM with LOD scores of 11.6 and 7.9, respectively. The development of an RFLP map has been much difficult in wheat than in most other crops due to the polyploidy nature of the crop, a high proportion of repetitive DNA, and unusually low levels of polymorphism within the genome (Chao *et al.*, 1989). Levels of polymorphism in wheat detected by RAPD analysis were similar to those detected by RFLP analysis (Devos and Gale, 1992).

In the present study, out of nine primers screened, only one RAPD markers was found to be linked in coupling to the monogenic line (Lr34/Yr18 region) transfer containing the gene for resistance to stripe rust. Similar results were reported for screening RAPD markers for linkage with the Lr24 gene introgressed from *Agropyron elongatum* into wheat. Only one RAPD marker, out of 360 primers tested was found to be linked to the Lr24 gene (Schachermayr *et al.*,1995). These problems may be related to the complex genome of wheat, which contains a large fraction of repetitive DNA (Sun *et al.*, 1997).

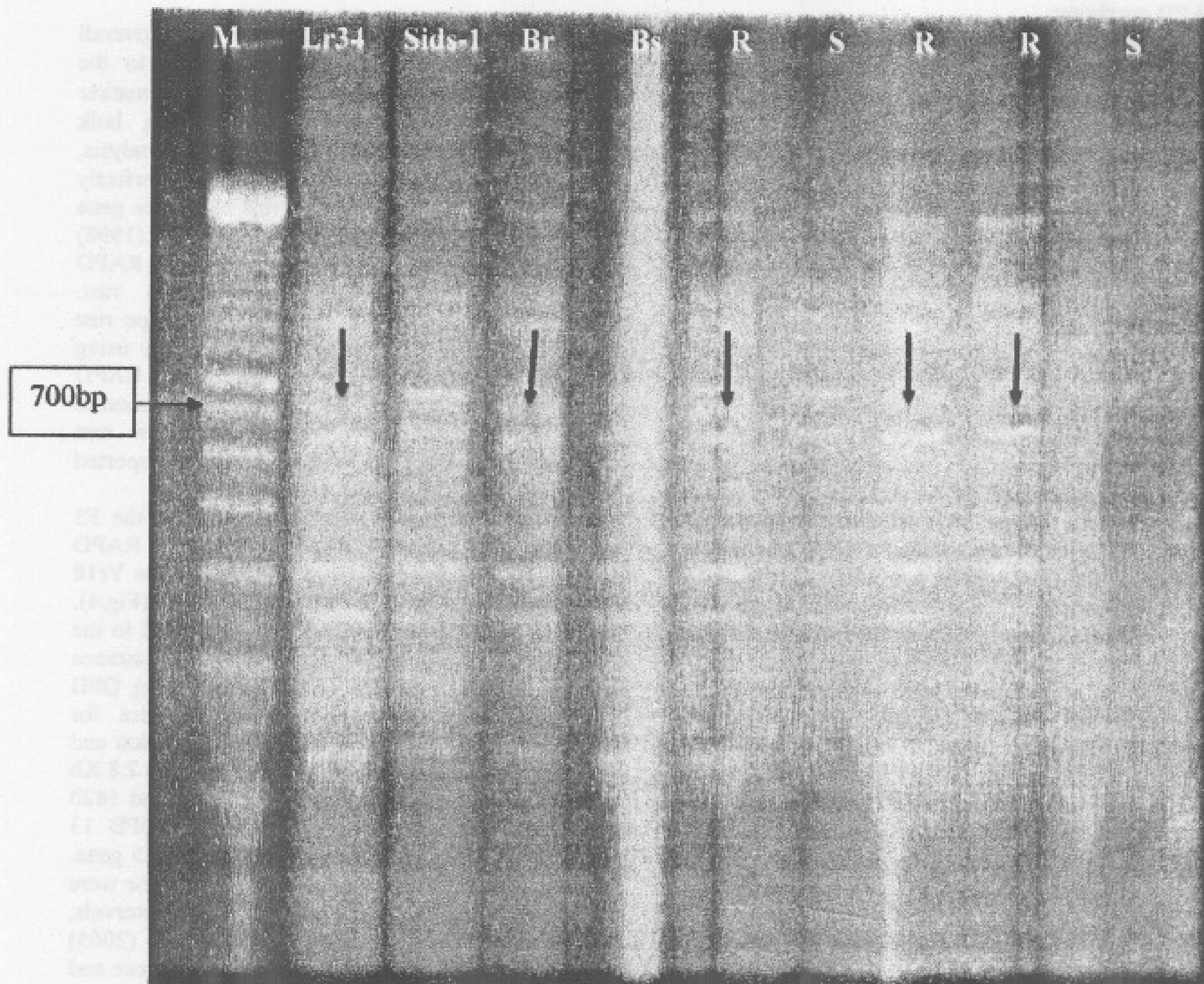

Fig.1: Amplification of a specific band produced by primer OPB13 (5'TTCCCCCGCT3'). Lanes: Br, Bulk resistance; Bs, Bulk susceptible; M, Molecular weight marker. R=resistant; S=susceptible. Arrow indicates the position of the specific band.

STS Markers:

The PCR conditions for STS analysis were optimized by investigating three factors, including primer concentration, primer annealing and the PCR programs. First, BF473324 primer assayed for its ability to reveal polymorphism between the monogenic line Lr34 and the cultivar Sids1. The tested concentrations of the primer BF473324 ranged from 0.2 to 1 μM are used. it was found that the primer concentration of 1μM gave the most reproducible amplification. However, no clear polymorphism was revealed. To improve the amplification and to obtain clear polymorphism , different PCR programmes with different annealing temperatures were tested. BF473324 primer did not reveal polymorphism with all tested programs with different annealing temperatures. Primer BE493812 had revealed polymorphism, when it was tested with G program (494C - 4min. ; 40 cycles (92 C- 1min ; 59C -1min. ; 72C - 2min) ; 72C - 5min).

The successful primer BE493812 was used to verify STS markers for Lr34/Yr18 region amplified PCR products from genomic DNA of the monogenic line Lr34/Yr18 and the wheat cultivar Sids1.The size of the amplified marker fragment was 250 bp using specific primer BE 493812. However, we were not able to amplify any DNA product using the specific primer BF473324. Amplification product of 250 bp, corresponding to EST-based STS primer was

identified in DNA extracts of the resistant parent Lr34/Yr18 as well as the resistant bulked DNA (Fig. 2). This band was absent in the susceptible parent " Sids 1" as well as the susceptible bulked DNA.

This polymorphic marker, primer BE 493812 (250 bp), was further used to check its linkage to the Lr34/Yr18, using the segregating F3 population from a cross between the resistant parent Lr34/Yr18 and the susceptible parent "Sids 1".For the EST-based STS marker primer BE493812 (250 bp), 59 of the 80 individuals in the population exhibited the amplified polymorphic fragment (250 bp), while the remaining did not (Fig. 3). The ratio fitted the expected Mendelian ratio, 3:1 (χ^2= 0.067, P< 0.01).

A regression analysis was performed to test the significance of the linkage between Lr34/Yr18 and the polymorphic markers. The results showed that the regression for the BE493812 (250bp) marker was significant. The calculated R^2 for BE493815 marker was 11%. This means that the relative contribution to the over all phenotypic variation among F3 plants due to the difference between alternative alleles at EST-based STS marker (250bp) locus was 11%.

After mapmaker linkage analysis on the F3 population, the genetic distance between STS marker (BE493812) and stripe rust resistance gene Lr34/Yr18 was determined to be 28.8 cM with the 2.2 LOD (Fig.4). Therefore, STS marker (BE493812 (250bp)) was linked to the quantitative trait loci (QTL) for stripe rust resistance gene Lr34/Yr18. Similar results were reported using BE493812 primer for mapping the durable resistance gene Lr34 in several spring wheat lines (Nelson *et al.*, 1997; Suenaga *et al.*, 2003). Recently, Schnurbusch *et al.* (2004) have improved the genetic map in the target region of QLr P.sfr-7DS using microsatellite and expressed sequence tag (EST) markers .They also reported that the Lr34/Yr18 region acts as a quantitative gene conferring durable leaf rust resistance, particulary in combination with other major resistance genes. The detection of STS markers for Lr34/Yr18 in our study indicated that STS markers for this resistance gene is highly specific and could be useful in wheat breeding programs. Several criteria such as linkage between the marker and the gene of interest, in order to avoid false positives, reliability and repeatability of the marker, as well as, the cost and reliability of field screening should be taken into account when DNA markers are considered for use in breeding programs (Schachermayr *et al.*, 1994; Koebner and Summers, 2002; Korzun, 2002).

Strategies using restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) have been used successfully to develop markers for some Lr resistance gene:Lr1,Lr9 Lr10, Lr13, Lr19, Lr23, Lr24, Lr25, Lr27, Lr28, Lr29, Lr31, Lr35, Lr37and Lr47 (Chelkowski and Stepien, 2001). For more efficient identification and more practical use , RFLP and RAPD markers have been converted into sequence-tagged sites (STS), sequence - characterized amplified regions (SCAR), or cleaved amplified polymorphic sequences (CAPS) (Autrique *et al.*,1995 ; Prins *et al* ., 1996). The first STS marker was developed by Schachermayr *et al.* (1994) for the gene Lr9 introgressed into *T. aestivum* from *Aegilops umbellulata*. Many wild relatives have been used to transfer resistance genes into wheat cultivars, with over 50 translocations of those genes recorded in the literature (Friebe *et al.*,1996). Several resistance genes such as Lr9,Lr19,Lr24,Lr26,Lr28, Lr35,Lr37,Lr39and Lr49 originating from wild relatives have been identified by STS, SCAR or CAPS markers (Scharchermayr *et al.*, 1994; Feuillet *et al.*,1995; Scharchermayr *et al.*, 1997). Three resistance genes Lr1, Lr10 and Lr21 have been cloned and their sequences are available (Ling *et al.*, 2002 ; Huang *et al.*, 2003).

RAPD and STS markers results revealed that Sids1 do not has Yr18 , when evaluated to stripe rust resistance at seedling stage. Moreover, identification and mapping of RAPD and STS markers linked to Lr34/Yr18, in our study, showed that Lr34/Yr18 involved in seedling plant resistance . Similar results were obtained by several investigators (Singh *et al.*, 2000 ; Imtiaz , 2002 ; Imtiaz *et al.* , 2004). They reported that DNA markers have been used to identify wheat chromosomal regions carrying stripe rust resistance genes effective at seedling and adult-plant stages.

The availability of such RAPD and STS markers should accelerate the introgression of the resistance gene into current wheat cultivars . It will also facilitate the accumulation of several resistance genes in a target cultivar. However, further efforts are needed to narrow down the marked interval spanning the target gene (Lr34/Yr18) Molecular markers which are closely linked with target alleles, present a useful tool in plant breeding since they can help to detect the resistant genes of interest without the need of carrying out disease tests,

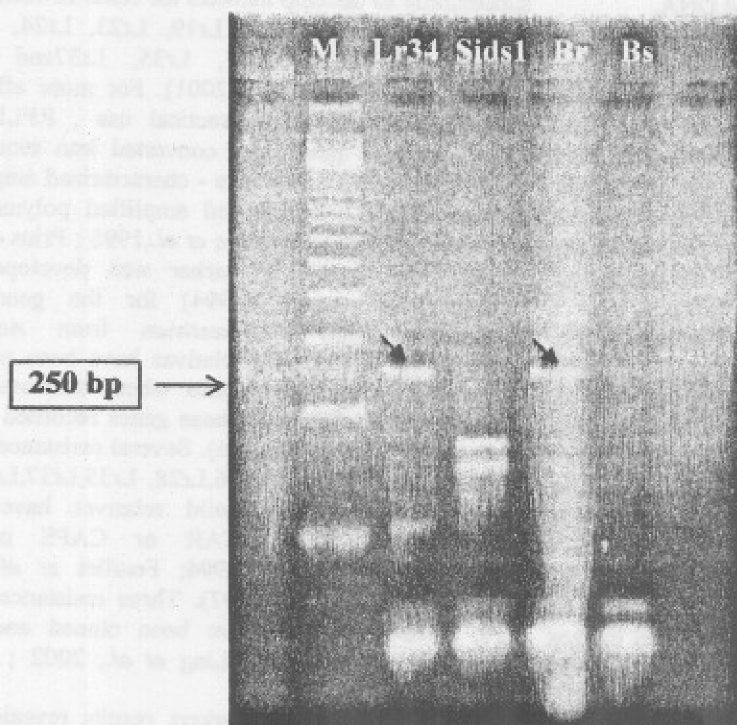

Fig 2: Amplification of a specific band produced by primer BE493812. Lanes: Lr34 (resistant parent) and Sids1(susceptible parent). Br, Bulk resistance; Bs, Bulk susceptible; M, Molecular weight marker. Arrow indicates the position of the specific band.

PCR- based RAPD markers are often inherited in a dominant. RAPD assay has the advantages that only minimal amounts of DNA are required, non radioactivity is used, and species- specific probes are not needed. Providing that a thermo cycling instruments is available, start-up costs for the RAPD assay are minimal. Furthermore, the exchange of RAPD information between laboratories is simple, as only the primer DNA sequence and the size of the polymorphic band need to be provided. RAPD analysis is fast and relatively economical, but the results of RAPD markers are often non-reproducible and population-dependent in many cases. The results of the present study indicate that DNA markers linked to the slow rusting genes Lr34/Yr18 and Lr46/Yr29 would facilitate the pyramiding of other leaf and yellow rusts resistance genes and laminates the time consuming progeny testing of individual plants in a breeding program. Also, these markers can be used on seedlings such as the population Lr34/Yr18 seedling, thus avoiding the lengthy of the generation disease testing time.

In this study we can suggest to the usage of the slow rusting gene pair Lr34/Yr18 in combination with other slow rusting genes in wheat cultivars as well as with the help of RAPD and STS markers to develop a new Egyptian wheat cultivars that will have a broad spectrum of resistance to leaf and stripe rust infection.

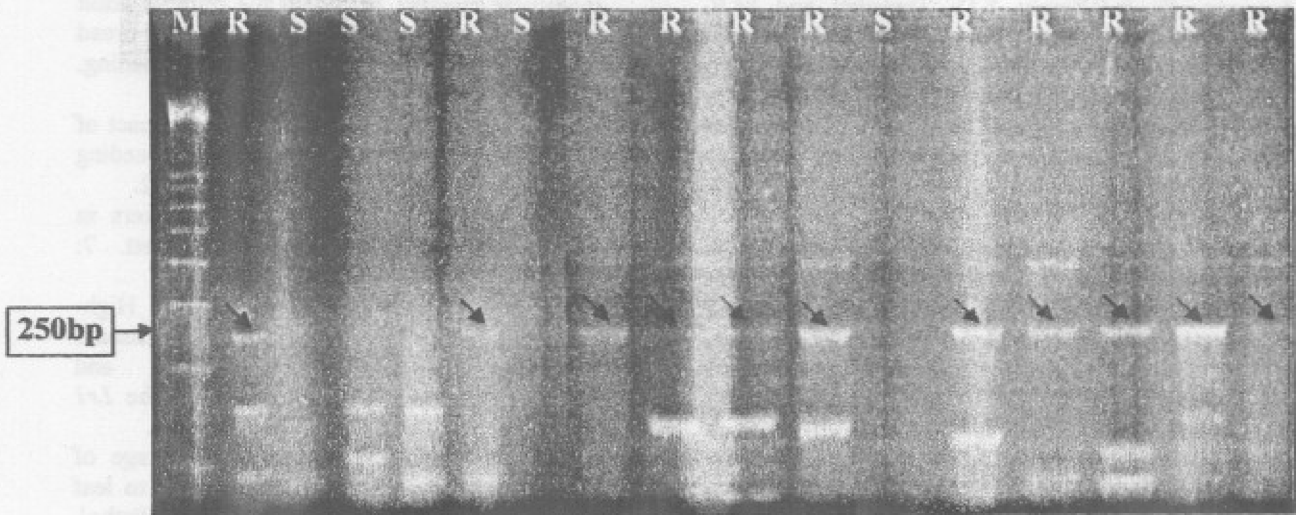

Fig 3: STS fragments produced by primer BE 493812, from left to right, Molecular weight marker followed by F3 individuals(R = resistant; S= susceptible). Arrow indicates the position of the specific band.

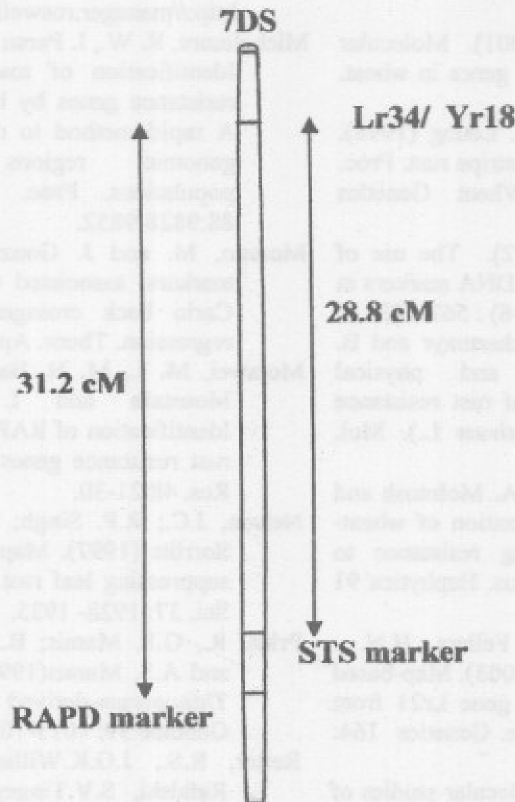

Fig 4: RAPD marker (primer 7_{700}) and STS marker (BE493812) were located through the MAPMMAKER - QTL analysis. ALL distance are given in CentiMorgan using Kosambi's mapping function.

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الملخص العربي

تحديد الدلائل الجزيئية المرتبطة بمنطقة المقاومة Lr34/Lr18 للصدأ الأصفر فى القمح

أ.د. محمد نجيب بركات ، د. هدى أحمد شكم ** ، د. محمد ابراهيم مطاوع* ، أ.د. محمد أحمد السهريجى* ،
د. عبد العزيز أبو على ***
* معمل التقنية الحيوية قسم المحاصيل ** قسم الوراثة – كلية الزراعة – جامعة الإسكندرية.
***محطة البحوث الزراعية بسخا– مركز البحوث الزراعية – القاهرة.

هدف هذه الدراسة هو تحديد RAPD markers و STS المرتبطة بجين المقاومة للصدأ الأصفر في عشيرة الجيل الثالث من القمح باستخدام طريقة Bulked Segregant Analysis (BSA) مع RAPD markers و STS . وقد تم استخدام العشيرة الناتجة من التهجين بين سدس ١ و Lr34 والتى تم تقويمها لمقاومة الصدأ الأصفر فى مرحلة البادرة. ولقد وجد بتحليل RAPD مع استخدام كل من BSA و برنامج Map manager أن الـ marker OPB13 700bp قد اظهر ارتباطا بالجين Lr34 بمسافة قدرها ٣١.٢ سنتيمورجان. (LOD = ٢,٣) .

وقد تم تحديد ظروف الـ PCR المثلى لبادئات STS المستخدمة مع الجين Lr34/Yr18 من خلال اختبار ثلاثة عوامل مؤثرة على نواتج الـ PCR وهى تركيز البادئ المستخدم وارتباط البادئ بالـ DNA والبرنامج المستخدم. وقد تم استخدام البادئ BE493812 فى عمل خريطة وراثية لمنطقة Lr34/Yr18 وذلك باستخدام Bulked Segregant Analysis (BSA) مع EST- based STS markers على نفس العشيرة الأخيرة. ولقد وجد بتحليل الارتباط باستخدام برنامج Map maker إن الـ marker BE493812 250 bp قد اظهر ارتباطا بالجين Lr34 بمسافة قدرها ٢٨,٨ سنتيمورجان . (LOD =2.2).

ويمكننا القول أن إتاحة استخدام مثل هذه الـ STS, RAPD markers يسهل من إدخال جينات المقاومة لأصناف القمح وأيضاً يسهل من تراكم بعض جينات المقاومة فى الصنف المستهدف تحسينه. وعلى الرغم من ذلك نوصى بمزيد من المجهودات للحصول على DNA marker على مسافة قريبة جداً من الجين 7DLr34/Yr18.