Turkish Journal of Field Crops, 2010, 15(1): 34-39

A PRELIMINARY STUDY FOR IDENTIFICATION OF CANDIDATE AFLP MARKERS FOR LEAF RUST RESISTANCE GENE *LR13* BY USING NEAR-ISOGENIC LINES OF SPRING WHEAT CV. THATCHER

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ABSTRACT

The aim of the present study was to detect candidate DNA markers for leaf rust resistance gene *Lr13*. The NIL groups were constructed from a set of near isogenic lines of wheat cv. Thatcher containing in total 41 different *Lr* resistance genes. A total number of 246 primer combinations in the NIL groups were screened for DNA polymorphism by using the AFLP method. Twenty-eight AFLP primer combinations yielded 33 polymorphic bands in Tc+Lr13 line. To confirm these polymorphic bands, 28 primer combinations were investigated in 41 Thatcher near isogenic lines. Six primer combinations amplified specific band products in Tc+Lr13. It was concluded that these AFLP markers can be linked to *Lr13* gene and in further studies on *Lr13* resistance gene, these six AFLP markers should be analyzed in near isogenic lines. Also, construction of the NIL groups can be useful in terms of time and cost of the molecular researches including Thatcher near isogenic lines.

Key words: AFLP, leaf rust, *Lr13*, wheat.

INTRODUCTION

Leaf rust caused by the pathogen *Puccinia recondita* Roberge ex Desmaz. f. sp. *tritici* (Eriks. & E.Henn) is the most common disease of wheat (*Triticum aestivum* L.) (Samborski, 1985). Crop losses up to 30% or more under favorable conditions can be serious. Grain yield losses are primarily caused by reduced floret set in severe epidemics (Roelfs et al., 1992). Yield losses can also result from reducing the rate of photosynthesis, increasing the rate of respiration and decreasing translocation of photosynthates from infected tissue (Roelfs et al., 1992; Agrios, 2005).

More than 50 different resistance genes against *Puccinia recondita* f. sp. t*ritici* have been reported for hexaploid wheat (McIntosh et al., 2003). Among the leaf rust (*Lr*) resistance genes identified, *Lr13* is probably the most widely distributed gene in the world (McIntosh et al., 1995). For example, most of the wheat varieties of the CIMMYT (Centro Internacional de Mejoramiento de Maizy Trigo, Mexico) possess *Lr13* resistance gene (Rajaram et al., 1988). Winzeler et al. (2000) identified *Lr13* resistance gene alone or in combination with other resistance genes in forty two of seventy two European wheat cultivars and breeding lines. *Lr13* is known as one of the adult plant resistance (APR) genes which are only effective in the adult stage (McIntosh et al., 1995). APR genes confer resistance to avirulent pathotypes only in post-seedling growth stages (Park and McIntosh, 1994).

Lr13, as a single gene, does not show resistance to leaf rust in most wheat-growing areas any more (McIntosh et al., 1995). However, *Lr13* is still an important resistance gene because in combination with other genes it provides protection against most pathotypes of leaf rust (Winzeler et al., 2000). Singh et al. (2001) reported that combination of genes *Lr1* and *Lr13* showed seedling resistance in an Australian wheat variety. The combination of *Lr13* and *Lr34* is the basis of most of the durable resistance in wheat (Roelfs, 1988).

Near-isogenic lines (NILs) carrying *Lr* resistance genes were obtained by using the spring wheat cv. Thatcher background (Dyck and Samborski, 1968; Chelkowski et al., 2003). Almost all genes are common in NILs developed through backcrossing, however they are differentiated only by the allelic composition at the interested locus and in the adjacent chromosomal region. Because of this, any polymorphism between two NILs at DNA level can be closely linked to target gene (Masocj, 2002). Thatcher-based NILs are the most valuable material in terms of identifying resistance genes (Winzeler et al., 2000) and pyramiding desired resistance genes into new cultivars (Chelkowski et al., 2003).

Different molecular marker strategies have been used to identify markers for *Lr13* gene such as in other leaf rust resistance genes. For example, restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) techniques were used in the identification of DNA markers for *Lr13* but no AFLP marker was linked to *Lr13* (Seyfarth et al., 2000). The AFLP technique was developed as a highly reproducible marker system by Vos et al. (1995) and it was used to tagging different leaf resistance genes in bread wheat. Blaszczyk et al. (2005) used ^{PstI}AFLP method and Thatcher near isogenic lines to detect candidate DNA markers for

some leaf rust resistance genes. They identified molecular markers for *Lr24*, *Lr26* and *Lr37* genes but failed in the

Table 1. Thatcher near-isogenic lines used in the present study.

Code Number	*Name*	**Pedigree**
1	Lr1	Tc*6/Centenario
2	Lr2a	Tc*6/Webster
3	Lr2b	Tc*6/Carina
4	Lr2c	Tc*6/Loros
5	Lr3	Tc*6/Democrat
6	Lr3bg	Bage/Tc*8
7	Lr3ka	Tc*6/Aniversario
8	Lr9	Transfer/Tc*6
9	Lr10	Tc*6/Exchange
10	Lr11	Tc*2/Hussar
11	Lr12	Exchange/Tc*6 APRes.
12	Lr13	Tc*6/Frontana
13	Lr14a	Selkirk/Tc*6
14	Lr14b	Tc*6/Mario Escobar
15	Lr15	Tc*6/Kental 483 (RL6052)
16	Lr16	Tc*6/Exchange
17	Lr18	Tc*7/Africa 43
18	Lr19	Tc*7/Translocation 4-*A. elongatum*
20	Lr20	Tc*6/Jimmer
21	Lr21	Tc*6/RL 5406 T.C.x *Ae. squarrosa*
22	Lr22	Tc*6/RL 5404 T.C.x *Ae. squarrosa*
23	Lr23	Lee 310/Tc*6
24	Lr24	Tc*6/Agent
25	Lr25	Tc*6/Transec.
26	Lr26	Tc*6/St-1-25
27	Lr28	Tc*6/C-77-1
28	Lr29	Tc*6/CS7D-Ag#11
29	Lr30	Tc*6/Terenzio
30	Lr32	Tc*6/3/*Ae. squarrosa*
31	Lr33	Tc*6/PI 58548 (1+gene)
32	Lr34	Tc*6/PI 58548 (2+gene)
33	Lr35	Tc*6/RL5711
34	Lr37	Tc*8/VPM
35	Lr38	Tc*6/T7 Kohn
36	Lr38	Tc*6/TMR-514-12-24
37	Lr44	Tc*6/*T. speltoides*
38	Lr44	Tc*6/8404
39	LrB	Tc*6/Carina
40	LrB	Tc*6/PI 268316
41	LrW	Tc*6/V336

The objective of this study was to detect candidate AFLP markers for *Lr13* resistance gene by using Thatcher near-isogenic lines. Firstly, AFLP analysis was run on the line Tc+Lr13 and the NIL groups constructed from Thatcher near-isogenic lines. Line specific bands were identified in Tc+Lr13 and these bands were investigated in all of the 41 Thatcher near isogenic lines.

MATERIALS AND METHODS

Plant material

Seeds of 41 Thatcher near-isogenic lines were kindly supplied by the CIMMYT, Ankara, Turkey. Leaf rust resistance genes and pedigree of all lines are given in Table1.

Template DNA Preparation

All laboratory studies were carried out in the laboratory of the Department of Plant Breeding, Institute for Agronomy and Plant Breeding I, Justus-Liebig University, Giessen, Germany.

Table 2. NIL groups, resistance genes and chromosome locations of NILs constituting NIL groups.

NIL Group	Resistance gene	Chromosome location
1	Lr11	2A
2	Lr17	2AS
	Lr37	2AS
3	Lr13	2BS
4	Lr16	2BS
	Lr23	2BS
	Lr35	2B
5	Lr1	5DL
	Lr2a	2DS
	Lr2b	2DS
	Lr2c	2DS
	Lr3	6BL
	Lr3bg	6BL
	Lr3ka	6BL
	Lr9	6BL
	Lr10	1AS
6	Lr12	4BS
	Lr14a	7BL
	Lr14b	7BL
	Lr15	2DS
	Lr18	5BL
	Lr19	7DL
	Lr20	7AL
	Lr21	1DS
	Lr22	2DS
7	Lr24	3D
	Lr25	4BS
	Lr26	1BL
	Lr28	4AL
	Lr29	7DS
	Lr30	4AL
	Lr32	3DS
	Lr33	1BL
	Lr34	7DS
8	Lr38	6DL
	Lr38	-
	Lr44	1B
	Lr44	-
	LrB	-
	LrB	-
	LrW	-

The seeds of 41 Thatcher NILs were sown and grown in pots in the greenhouse. DNA was extracted from fresh leaves tissue according to a standard CTAB method described by Doyle and Doyle (1987).

To find more AFLP markers linked to *Lr13* resistance gene, the NIL groups were made from DNA samples of Thatcher NILs after DNA dilution. At the beginning of this study, identification of candidate AFLP markers for *Lr11* and *Lr13* resistance genes was planned. Therefore, Thatcher NIL groups were made on the basis of *Lr11* and *Lr13* resistance genes. The NIL groups, resistance genes of the lines, which

Table 3. Primer combinations showed products specific to line Tc+Lr13 in NIL groups.

No.	*Primer*	*Selective bases*	*No.*	*Primer*	**Selective bases**
1	E31 M56	AAA-3' CGC-3'	15	E45 M67	ATG-3' GCA-3'
2	E32 M53	AAC-3' CCG-3'	16	E45 M68	ATG-3' GCC-3'
3	E32 M56	AAC-3' CGC-3'	17	E47 M64	CAA-3' GAC-3'
4	E32 M63	AAC-3' GAA-3'	18	E47 M68	CAA-3' GCC-3'
5	E32 M69	AAC-3' GCG-3'	19	E47 M69	CAA-3' GCG-3'
6	E32 M74	AAC-3' GGT-3'	20	E49 M67	CAG-3' GCA-3'
7	E33 M63	AAG-3' GAA-3'	21	E49 M68	CAG-3' GCC-3'
8	E34 M70	AAT-3' GCT-3'	22	E51 M63	CCA-3' GAA-3'
9	E34 M71	AAT-3' GGA-3'	23	E51 M80	CCA-3' TAC-3'
10	E39 M57	AGA-3' CGG-3'	24	E52 M63	CCC-3' GAA-3'
11	E41 M70	AGG-3' GCT-3'	25	E56 M71	CGC-3' GGA-3'
12	E43 M74	ATA-3' GGT-3'	26	E57 M69	CGG-3' GCG-3'
13	E43 M75	ATA-3' GTA-3'	27	E57 M74	CGG-3' GGT-3'
14	E45 M66	ATG-3' GAT-3'	28	E58 M67	CGT-3' GCA-3'

constructed the groups, and chromosome location of resistance genes were given in Table 2. Firstly, Tc+Lr11 was present in group 1 and *Lr11* gene is located on wheat chromosome 2A. The lines which possess resistance genes located on the same chromosome (2A) were put in group 2. Same process was applied to *Lr13* gene in group 3 and 4. Other Thatcher NILs were distributed among group 5, 6, 7 and 8 without any common speciality. Except group 1 and 3, the number of lines in each group varied between 2 and 9.

Digestion, adapter ligation, preamplifications and selective amplification were carried out according to the methods previously described (Zabeau and Vos, 1993; Vos et al., 1995). DNA restriction and ligation were performed by using the AFLP Core Reagent Kit (Invitrojen, Germany). The amount of 150 ng DNA was digested with 2.5 U *Eco*RI and *Mse*I for 2 h at 37°C and 15 min at 70°C in 1 X Buffer (50 mM Tris-HCl, pH 7.5, 50 mM Mg- acetate, 250 mM K-acetate) in a total volume of 25 µL. Adapters of known sequences were ligated to the ends of restricted fragments by using adapter ligation solution (*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) and T4 DNA ligase for 2 h at 20°C. Ligation was followed by two preamplification steps.

+0 Preamplification

Ligated DNA was diluted 10 times by adding TE buffer (10 mM Tris, pH: 8.0, 1 mM EDTA, pH: 8.0) and used as a template for the +0 amplification. Non-selective AFLP primers E00 and M00 were used in order to reduce unspecific background on polyacrylamide gel. The PCR reaction was performed in a total volume of 50 µL containing 5.0 µL of diluted DNA, 75 ng of each primer (MWG Biotech), 0.2 mM dNTP mix (PeQlab Biotech. GmbH), 1 U Taq DNA polymerase (Eppendorf Brinkmann Ins. USA) and its corresponding reaction buffer. The amplification was carried out under the following programme conditions: 3 min at 94°C-initial denaturation, 20 cycles of 30 s at 94°C, 1 min at 56°C, 1 min 72°C with final extension of 5 min at 72°C.

+1 Preamplification

After +0 preamplification, DNA was diluted 10 times with TE buffer again. The amplification was carried out with same PCR components and conditions as such in +0 preamplification. E01, E02, M02 and M03 primers carrying one additional selective nucleotide were used distinctly.

+3 Amplification

The PCR reaction of the +1 preamplification was diluted 10 times with TE buffer in order to use DNA template in selective amplification (+3 amplification). In each case the *Eco*RI primer was labelled at the 5'-end with fluorescence dye IRD700 or IRD800 (MWG Biotech). The selective PCR was performed in a total volume of 20 µL that contained 5.0 µL of diluted DNA, 30 ng *Mse*I+3 primer, ng 5'-IRD700-labelled *Eco*RI+3 primer or 10 ng 5'-IRD800-labelled *Eco*RI+3 primer, 0.2 mM dNTP mix (PeQlab Biotech. GmbH) and 0.5 U Taq DNA polymerase (Eppendorf Brinkmann Ins. USA) and its corresponding reaction buffer. The cycle profile for selective amplification was as follows: 3 min at 94°C-initial denaturation, 12 cycles of 30 s at 94°C, 30 s at 65°C (-0.7°C/cycle), 1 min at 72°C and 22 cycles of 30 s at 94°C, 30 s at 56°C, 1 min at 72°C with final extension of 5 min at 72°C. All PCR reactions were carried out in a GeneAMP PCR System 9700 (Perkin-Elmer).

Electrophoresis

An equal volume of formamide loading buffer (% 96 formamide, 10 mM EDTA pH 8.0, % 0.1 fuchsin) was added to the samples and denatured at 94°C at 1.5 min. A 25 cm, 8% denaturing polyacrylamide gel (Long Ranger) was prepared and preheated for 30 min. 1.0 µL of each samples was loaded on to the gel and electrophoresis was conducted in 1 x Long Run TBE buffer at 1.500 V, 40 W, 40 mA and 48°C using a Li-Cor DNA Gene Readir 4200 (MWG Biotech. Ebersberg/Germany). The 50 to 700 bp standard ladder (IRDye 700) was used for determination of the generated fragment sizes.

RESULTS

Two hundred forty six selective primers were tested on NIL groups. Each primer combination produced AFLP bands ranging from 65 to 98 and the total number of bands obtained was approximately 20.000. Size of bands varied from 40 to 650 bp.

Using NIL groups, 28 of 246 primer combinations produced line specific bands in near isogenic line Tc+Lr13 (Table 3). Due to the observing more polymorphic band patterns in line carrying *Lr13*, *Lr11* was not studied after that. Twenty eight AFLP primer combinations displayed totally 33 polymorphic bands.

Polymorphic band number per primer combination varied from one to three. Except E32 M63, E34 M71, E47 M64 and E57 M69, each of the other primer combinations produced only one polymorphic band. Two polymorphic bands were observed in E32 M63, E34 M71 and E47 M64 combinations while E57 M69 indicated three different polymorphic band patterns. One of these polymorphic bands was shown in Figure 1. E45 M67 primer combination generated a polymorphic band displaying a 340-bp DNA fragment specific for line Tc+Lr13 which was absent in NIL groups (Figure 1).

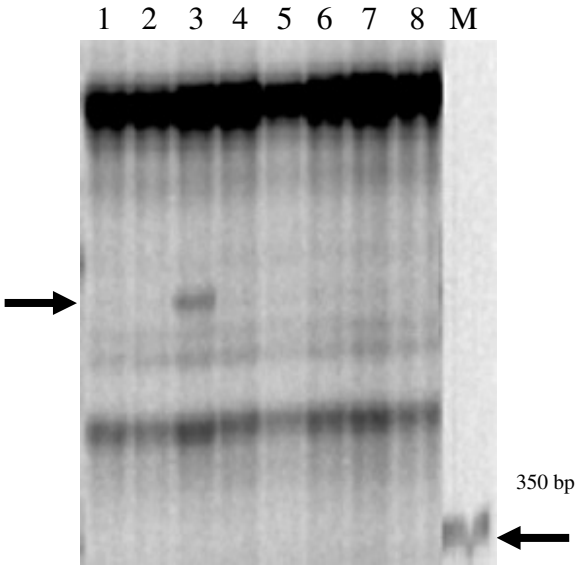

Figure 1. Partial AFLP profile using the *Eco*RI primer E45 and the *Mse*I primer M67, illustrating the polymorphic band (indicated by the *arrow*). Lanes 1-8: Thatcher near isogenic line groups (according to Table 2). Lane M is molecular weight marker, IRDye 700.

All twenty eight primer combinations, produced polymorphic bands in Tc+Lr13, tested in 41 Thatcher near isogenic lines. Six of twenty eight AFLP primer combinations E34 M70, E43 M75, E45 M67, E47 M64, E49 M67 and E57 M69 generated six polymorphic products in line Tc+Lr13, which were absent in other Thatcher near isogenic lines. One example was shown in Figure 2. E45 M67 primer combination amplified same polymorphic band in Tc+Lr13 but other lines did not have the band (Figure 2). Other twenty two primer combinations yielded non-specific amplification products for *Lr13*. Most of them amplified the polymorphic bands interestingly in lines Tc+Lr19 and Tc+Lr26, too.

Six polymorphic AFLP primer combinations produced differently sized amplified bands in line Tc+Lr13. Each of E34 M70, E43 M75, E45 M67 and E49 M67 combinations generated one polymorphic band of about 145 bp, 350 bp, 340 bp and 255 bp, respectively. E47 M64 and E57 M69 primer combinations, which had amplified more than one polymorphic band in line Tc+Lr13 using NIL groups, displayed one polymorphic band each displaying about 364 bp and 255 bp DNA fragment, respectively in line Tc+Lr13 using NILs.

DISCUSSION

Leaf rust resistance gene *Lr13* was screened for DNA polymorphism by using 246 AFLP primer combinations in Thatcher NIL groups and these number of primer combination are rather high. Blaszczyk et al. (2005) succeeded in identification candidate AFLP markers for some leaf rust resistance genes but failed for *Lr1, Lr9, Lr10, Lr13, Lr19, Lr21, Lr28* and *Lr35* genes and they concluded that screening low number of loci was one of the reasons of this failure. Since they tested 33 selective primers and obtained totally 286 bands. Seyfarth et al. (2000) used RFLP, SSR and AFLP markers to develop a linkage map around *Lr13* and a molecular marker for this resistance gene in two segregation populations. In both populations, RFLP probes and SSR were mapped proximal to *Lr13* and the microsatellite GWM630 was the closest molecular marker at 10 cM from *Lr13*. No AFLP product which was amplified by using 226 primer combinations was linked with *Lr13* in both populations.

The AFLP approach has been widely used for developing polymorphic markers in a number of crop plants, including bread wheat. A comparison of RFLP, RAPD (Random Amplified Polymorphic DNA) and AFLP mapping techniques for their relative efficiency in detecting polymorphism indicated that AFLP was the most efficient (Gupta et al., 1999). The results of the AFLP analysis displayed very high of monomorphism level in Thatcher near isogenic lines. Therefore, the presence of any polymorphic band in a near isogenic line can be due to the resistance gene in this line. Seyfarth et al. (2000) stated that NILs represent some of the best material for finding molecular markers linked to the gene of interest in spite of the low level polymorphism. Indeed, NILs have been successfully used to develop markers for leaf rust resistance genes in wheat, e.g. for the *Lr9, Lr10, Lr19, Lr24, Lr37* and *Lr47* (Chelkowski et al., 2003) or for the *Lr28* (Naik et al., 1998).

Michelmore et al. (1991) developed bulk segregant analysis (BSA) for identifying markers in specific region of the genome. BSA involves comparing two pooled DNA samples of individuals from a segregation population originating from a single cross. Individuals within each pool are identical for the trait or gene of interest but arbitrary for all other genes. In this experiment, NIL groups were used; they carry different near isogenic lines and thus *Lr* genes. We firstly searched any polymorphism in NIL groups by using 246 different primer combinations afterward polymorphic primers (28 of them) were tested in near isogenic lines.

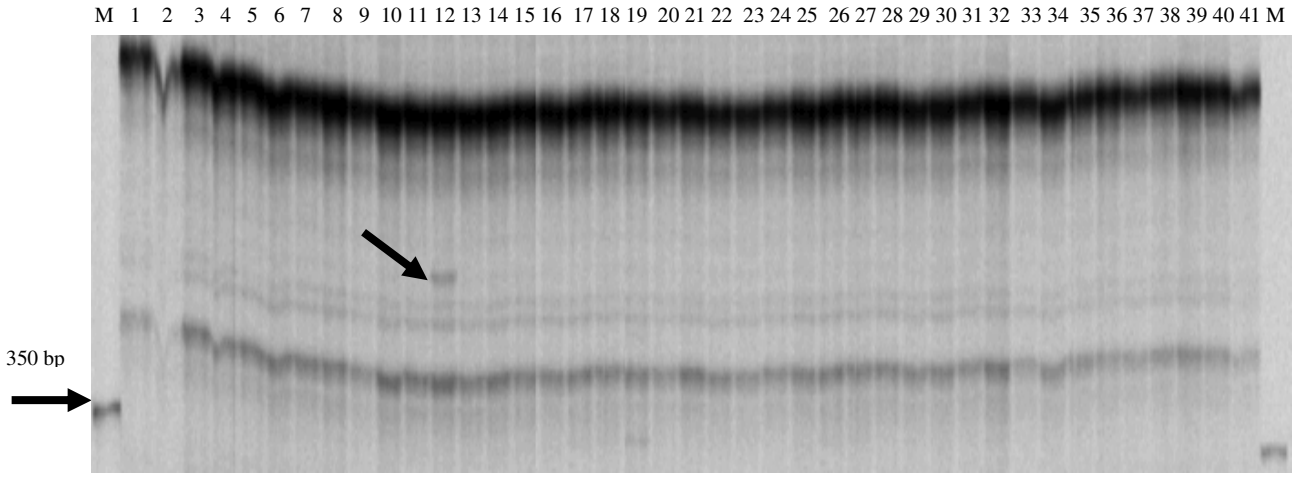

Figure 2. Partial AFLP profile using the *Eco*RI primer E45 and the *Mse*I primer M67, illustrating the polymorphic band (indicated by the *arrow*). Lanes 1-41: Thatcher near isogenic lines (according to Table 1). Lane M is molecular weight marker, IRDye 700.

To our knowledge, this is the first publication that report construction of NIL groups from Thatcher near isogenic lines. This method allowed screening more primer combination in a shorter time. Consequently, such an application would be useful in terms of time and cost of the molecular researches including Thatcher near isogenic lines.

In this study, six AFLP primer combinations (E34 M70, E43 M75, E45 M67, E47 M64, E49 M67 and E57 M69) were determined as candidate AFLP markers for *Lr13* resistance gene. Future studies should consider using these AFLP markers in a segregation population such as F_2 or they can be tested in genotypes carrying *Lr13* and convert into other marker systems like SCAR.

The results of the present study on the identification candidate markers for gene *Lr13* showed that the AFLP method is useful to detect candidate markers for leaf rust resistance genes. It was also concluded that Thatcher near isogenic lines which contain different resistance genes have a narrow genetic frame, so, they could provide well focusing to identify the interested resistance gene.

ACKNOWLEDGEMENTS

This research is part of PhD thesis of Fatma AYKUT TONK. We would like to thank Prof. Dr. Dr. h.c. Wolfgang FRIEDT for use of their laboratory facilities. We are grateful to Dr. Carola Wagner, Dr. Markus Rhiel and Annette Plank for their technical assistance.

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