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Original Article/Orijinal Makale

SCREENING OF *LR* GENES PROVIDING RESISTANCE TO LEAF RUST IN WHEAT USING MULTIPLEX PCR METHOD

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Abstract: Leaf rust is a fungal disease in wheat that causes significant decrease in yield around the world. In Turkey, several genes, including *leaf rust-resistant* (*Lr*) *Lr9, Lr19, Lr24* and *Lr28*, have been found to induce disease resistance. To obtain resistant cultivars during the breeding process, screening of these genes in various specimens is crucial. Thus, we aimed in the present study primarily to improve the multiplex polymerase chain reaction (PCR) methodology by which four *Lr* genes could be simultaneously screened in plant samples carrying these genes. Serial PCR experiments were carried out for determination of optimal PCR conditions for each *Lr* gene and in all studies nursery lines were used. PCR conditions were determined as follows: 35 cycles of 95°C for denaturation (30 s), 58°C for annealing (30 s) and 72°C for elongation (60 s), with an initial 94°C denaturation (3 min) and a 72°C extension (30 min). The primers used in the PCR runs were as follows: *Lr9*F: TCCTTTTATTCCGCACGCCGG, *Lr9*R: CCACACTACCCCAAAGAGACG; *Lr19*F: CATCCTTGGGGACCTC, *Lr19*R: CCAGCTCGCATACATCCA; *Lr24F*: TCTAGTCTGTACATGGGGGC, *Lr24*R: TGGCACATGAACTCCATACG; *Lr28*F: CCCGGCATAAGTCTATGGTT, *Lr28R*: CAATGAATGAGATACGTGAA. We found that the optimum annealing temperature for all four genes was 61°C and extension temperatures were 62°C or 64°C. Finally, using this new PCR method, we successfully screened these genes in specimens carrying only one single *Lr* gene. Optimal multiplex PCR conditions were; denaturation at 94°C for 1 min, 35 extension cycles [94°C for 30 s, 57–61ºC (ideal 61°C for 30 s), and 64–68°C for 2 min] and final extension at 72°C for 30 min. In addition, we achieved positive results when running the optimised multiplex PCR tests on *Lr19*, *Lr24* and *Lr28*. Future studies are planned to expand new wide multiplex PCR method to include all other *Lr* genes.

Key words: Cereals, *Puccinia*, disease, molecular-assisted selection, breeding.

Buğdayda Kahverengi Pas Hastalığına Dayanıklılığı Sağlayan Bazı *Lr* Genlerinin Multipleks PZR Metodu ile Taranması

Özet: Kahverengi pas buğdaylarda önemli bir fungal hastalık olup bütün dünyada önemli verim kayıplarına neden olmaktadır. Türkiye'de yapılan çalışmalarda *Lr9, Lr19, Lr24* ve *Lr28* gibi çeşitli *Lr* genlerinin bu hastalığa dayanıklılığı sağladığı tespit edilmiştir. Bu genlerin çeşitlerde ve örneklerde ıslah süresince başarılı bir şekilde taranması önemlidir. Bu nedenle; çalışmada esasen bu 4 geni içine alacak şekilde multipleks polimeraz zincir reaksiyonu (PZR) metodolojisinin başarılı bir şekilde tek seferde uygulanabileceği bir yöntemin oluşturulması amaçlanmıştır. Çalışmada her bir gen için referans oluşturacak şekilde pas kapan nörseri örnekleri kullanılmış ve ön denemelerdeki PZR şartları şu şekildedir: başlangıç denatürasyon initial 94°C'de 3 dk, denatürasyon 95°C'de (35 siklus, 30'ar sn), bağlanma (annealing) 58°C'de 30 s, uzama (elongation) 72°C'de 60 s ve son uzatma (final extension) 72°C'de 30 dk. olmak üzeredir. Primerler: *Lr9*F: TCCTTTTATTCCGCACGCCGG, *Lr9*R: CCACACTACCCCAAAGAGACG; *Lr19*F: CATCCTTGGGGACCTC, *Lr19*R: CCAGCTCGCATACATCCA; *Lr24*F: TCTAGTCTGTACATGGGGGC, *Lr24*R: TGGCACATGAACTCCATACG; *Lr28*F: CCCGGCATAAGTCTATGGTT, *Lr28*R: CAATGAATGAGATACGTGAA. Genelde tüm genler için ortak olmak üzere optimum bağlanma sıcaklığının, 61°C, uzatma sıcaklığının 62°C or 64°C olduğu tespit edilmiştir. Sonuç olarak, yeni PCR metodu ile tek gel taşıyan örneklerde başarılı bir şekilde taranma yapılmıştır. Optimal Multipleks PCR koşulları: denatürasyon 94°C'da 1 dk., 35 siklus [94°C'da 30 s., 61°C'da 30 s annealing ve 64–68°C'de 2 dk. extention] ve final extension 72°C'de 30 dk. olarak tespit edilmiştir. İlaveten; optimize multipleks PCR testleri ile *Lr19, Lr24* ve *Lr28* taşıyan çok genli örneklerde pozitif sonuçlar da alınmıştır. Çalışmanın bundan sonraki diğer *Lr* genleri ile büyütülmesi hedeflenmektedir.

Anahtar Kelimeler: Tahıllar, *Puccinia*, hastalık, moleküler destekli seleksiyon, ıslah.

Introduction

Leaf rust (*Puccinia triticina*) is a fungal disease that causes extensive losses in wheat production on a worldwide scale. Leaf rust also causes great environmental problems in terms of sustainable agriculture. Many farmers should apply fungicides twice, sometimes three times a year to prevent the development of this disease. An epidemic leaf rust case occurred in 9 million hectares of farmland in South America, and nearly $50 million of agricultural fund was needed tocombat the problem (Germán et al., 2007; Vanzetti et al., 2011). The most environmental-friendly and cost-effective way to

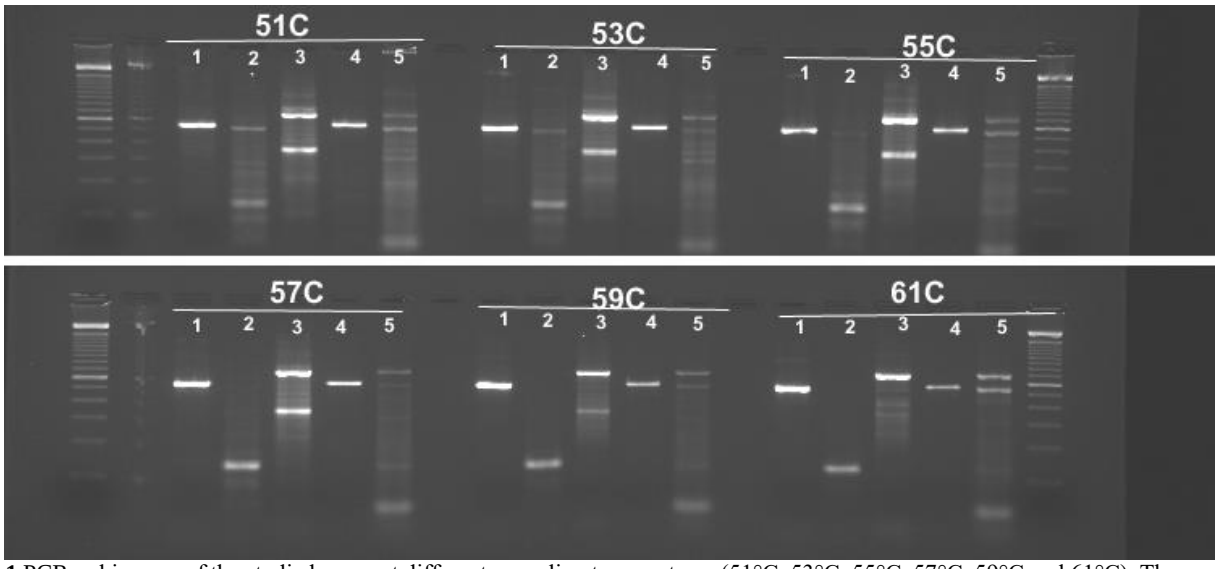

Fig 1 PCR-gel images of the studied genes at different annealing temperatures (51°C, 53°C, 55°C, 57°C, 59°C and 61°C). The sample order for each temperature was 1. *Lr9 2. Lr19 3. Lr24 4. Lr28* and 5. a DNA mixture from 4 different plants Ladder: 100 bp.

combat the disease is the use of resistant cultivars carrying leaf rust-resistant (*Lr*) genes (Li et al., 2007). The development of these cultivars is possible by means of successful breeding methods. *Lr9, Lr19, Lr24* and *Lr28* are some of the genes that provide resistance to the disease in Turkey (Anonym, 2015). No multiplex PCR studies have been published so far to screen for the presence of these genes in a single experiment to reduce the necessary labour, supplies and time for selection and breeding purposes. Therefore, we aimed to create a simple, one-step PCR methodology using a multiplex primer mix to screen for the presence of four *Lr* genes (*Lr9, Lr19, Lr24* and *Lr28*) in plant samples, to increase the success of wheat breeding and selection. The goal was to develop a multiplex PCR method which can screen multiple genes simultaneously in plant samples. The present study includes preliminary results towards these aims.

Materials and Methods

At the initial stages of the study, PCR experiments targeting a single gene were performed. Materials used as reference are as follows: *Lr9*: KHPKN, RL6010; *Lr19*: KHPKN, Tc*7/Transl. 4-A.elongatum; *Lr24*: KHPKN Tc*6/Agent, *Lr28*: KHPKN, Tc*6 /C-77-1. Leaf samples from plants were taken after seed germination under field conditions and frozen in liquid nitrogen. The samples were digested using special beads with the tissue lyser LYS (Life Science, USA). DNA was purified from the resulting powdered material using the DNeasy® Plant Mini Kit according to the protocol (Qiagen, 2015). In this protocol, the DNA material was treated with lysis buffer and the plant material was subjected to pre-column purification for DNA extraction. Then, the column was washed and the DNA lysate passed through the column filter. After this process, the column was washed again with buffer AE, and the DNA was eluted into the tube. The primers used in the PCR runs were as follows:

*Lr9*F: TCCTTTTATTCCGCACGCCGG,
*Lr9*R: CCACACTACCCCAAAGAGACG;
*Lr19*F: CATCCTTGGGGACCTC,
*Lr19*R: CCAGCTCGCATACATCCA;
*Lr24*F: TCTAGTCTGTACATGGGGGC,
*Lr24*R: TGGCACATGAACTCCATACG;
*Lr28*F: CCCGGCATAAGTCTATGGTT,
*Lr28*R: CAATGAATGAGATACGTGAA.

Positive and negative control assays of the primers were performed using the Thermal cycler (Applied Biosystems). The PCR cycle consisted of 35 cycles of 95°C for denaturation (30 s), 58°C for annealing (30 s) and 72°C for elongation (60 s), with an initial 94°C denaturation (3 min) and a 72°C extension (30 min). Initially, the total PCR volume was 25 µl, including 50 ng

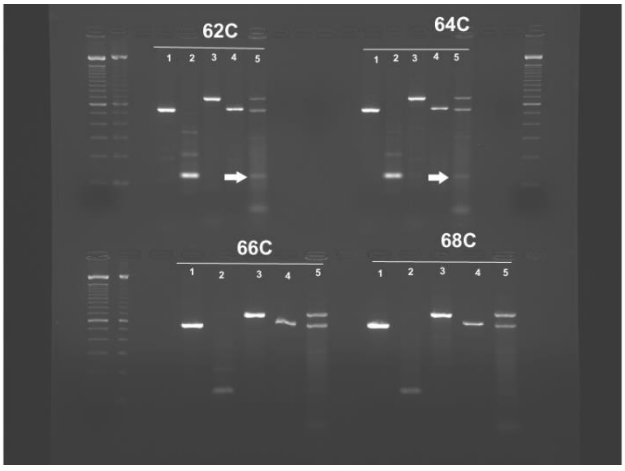

Fig. 2 PCR-gel images of the studied genes at different extension temperatures. Samples for each temperature were in the following order: 1. *Lr9* 2. *Lr19* 3. *Lr24* 4. *Lr28* and 5. a DNA mixture of 4 different plant samples. *Lr24, Lr28,* and *Lr19* (weak band, arrows) were seen in the 5th well, particularly at 62°C and 64°C. Ladder: 100 bp.

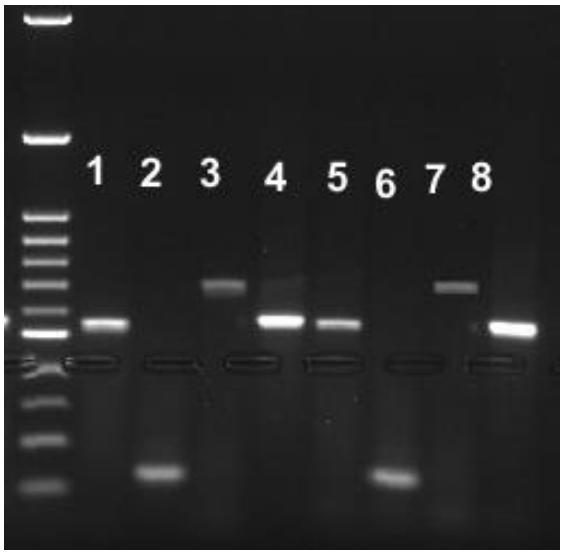

Fig. 3 The first four wells (1-4) show the results of normal PCR runs in which each gene was amplified only with its specific primer, The genes are, starting from the left side after the ladder, *Lr9*, *Lr19*, *Lr24*, *Lr28*, respectively. The next four wells (5-8) show PCR results for each gene treated with the multiplex primer mix with the same order of *Lr9*, *Lr19*, *Lr24*, *Lr28*. Ladder: 100 bp.

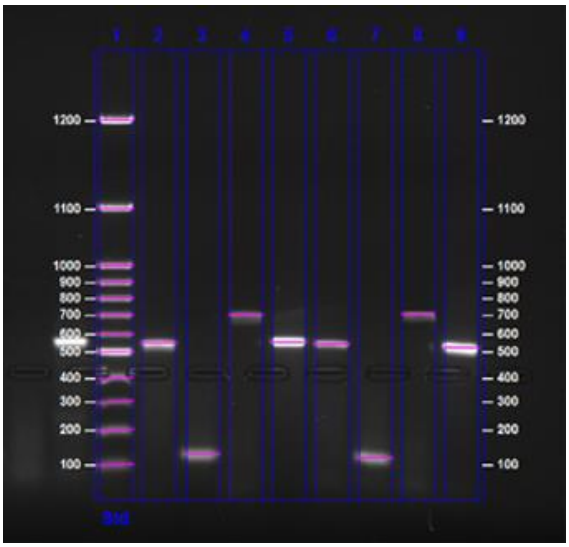

Fig. 4 Analysis of Fig. 3; 1. The first four lanes show normal PCR experiments with only one primer pair each; 2. a band at 547, 7 bases (*Lr9*), 3. a band at 123, 1 base (*Lr19*), 4. a band at 700, 0 base (*Lr24*) and 5. a band at 552, 7 bases (*Lr28*). The next lanes show PCR trials with a multiplex primer mix; 6. a band at 542, 8 bases (*Lr9*), 7. a band at 114, 9 bases (*Lr19*), 8. a band at 689, 2 bases (*Lr24*), and 9. a band at 523, 3 bases (*Lr28*). Ladder: 100 bp.

DNA, 1 × PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.8 and 0.1% Triton X-100), 200 µM deoxynucleotide triphosphates (dNTPs, Sigma Inc.), 120 nM of each primer (Life Science), 16 µl Sigma DNAse and RNAse-free water and 1 U Taq polymerase (Life Science). After optimising the target gene primers, the common annealing and extension temperatures, reaction times and DNA concentrations for all four genes experiments were conducted. So; for determination of optimal annealing temperature, samples containing *Lr9*, *Lr19*, *Lr24*, *Lr28* and DNA mixtures of plants carrying these four genes were tested at temperatures of 51°C, 53°C, 55°C, 57°C, 59°C and 61°C using gradient PCR: 94°C, 1 min denaturation, [94°C, 30 s, annealing temperatures (51°C, 53°C, 55°C, 57°C, 59°C, 61°C), extension 72°C, 2 min] × 35 cycles, final extension 30 min, 72°C. Regarding extension temperature optimizations, samples were run at the same order as in the annealing temperature experiments; 94°C, 1 min denaturation, [94°C 30 s, 61°C 30 s (annealing), 62°C, 64°C, 66°C, 68°C, 2 min (extension)]×35 cycles, final extension 30 min 72°C PCR reactions were performed. Determination of appropriate multiplex PCR conditions: after a series of trials, we determined the optimum conditions for PCR with multiplex primer mixes. The optimum primer concentration and DNA concentration were 5 nmol and 40 ng/µl, respectively, and the optimum PCR conditions were 95°C denaturation for 1 min, 35 extension cycles of 95°C for 30 s, annealing at 58°C for 30 s and extension at 64°C for 30 s, with a final extension at 72°C for 15 min. The total PCR volume in the tube was 50 µl, including 2 µl DNA template, 2 µl primer mix in total, 20 µl water and 25 µl master mix. The PCR products were run in an agarose gel prepared with 1.5 × TAE buffer for 90 min at 75 V. After PCR conditions were optimized for each of the four genes, samples with only one gene were scanned using the multiplex PCR method. In addition, DNA of three or four samples carrying different single genes was subjected to the multiplex PCR. Following all the PCR assays, 20 µl of the PCR products was added to 3 µL dye (R0611, Fermentas), 2 × TAE buffer (1.6 M Tris, 0.8 M acetic acid and 40 mM EDTA) with ethidium bromide and loaded onto a 2% agarose gel. Gene Ruler plus 100 base pairs (bp) DNA was used as the standard (Fermentas, SM0321). Bands were screened by using a UV-imaging system (BioRad chemoluminescence) and analyzed with the integrated software.

Results and Discussion

Annealing temperature optimisation studies

In summary, we found that the optimum annealing temperature for all four genes was 61°C since the formation of nonspecific bands of *Lr24* (lane 3) were blocked at this temperature (Fig. 1).

Extension temperature optimisation studies

Our results showed that the extension temperatures of 62°C and 64°C were more successful compared to our previous experiments and that multiplex assays at these temperatures (represented by every 5th well in the gel) showed prominent bands representing *Lr19*, *Lr24* and *Lr28* genes (Fig. 2). Our new PCR-optimization results and PCR conditions reported in other studies are compared in Table 1.

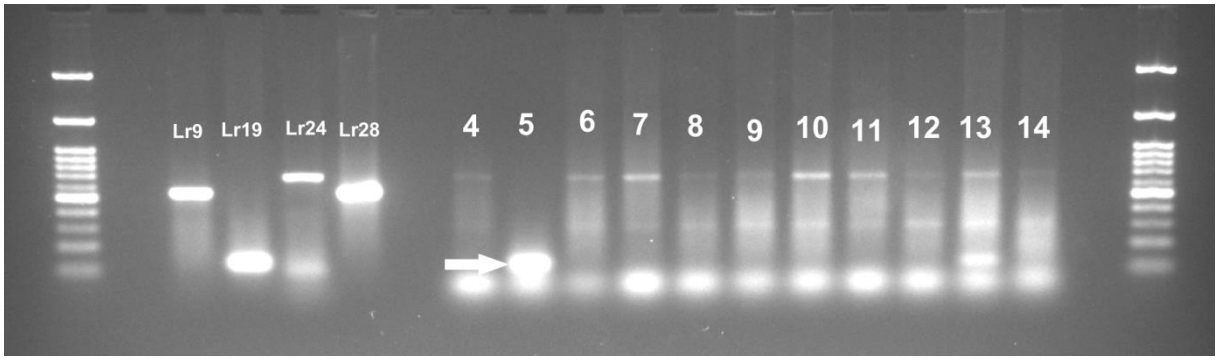

Fig. 5 In the first four wells, the reference bands of the PCR with the multiplex primer mix were observed. Numbers 4–13 represent different hybrid samples; in well 5, *Lr19* is marked with an arrow, while in well 13 both *Lr19* and *Lr24* are present. In other samples, only *Lr24* was found. Ladder: 100 bp.

Determination of appropriate multiplex PCR conditions (Fig. 3)

Analysis of the image in Fig. 3 is shown in Fig. 4, and sizes of all bands in the single and multiplex PCR trial were obtained as expected.

Gene screening in plant samples using the new multiplex PCR method

PCR conditions given in literature for each gene were different from our present results with which we determined as optimum conditions (Table 1). In a single-tube PCR reaction with a primer mix, the results can show multiple bands which may mislead the researcher. In addition, primer binding might be non-specific. As suggested by Henegariu et al. (1997) and Henegariu (2015), to avoid this problem, a series of attempts have been made to optimise the extension conditions, the annealing temperature and duration and the amount and concentration of primers and DNA template.

In this preliminary study, different genes from DNA mixtures prepared from different plants were subjected to the multiplex PCR with some successful results (Figs. 2, 5–6). In particular, our PCR trials with a multiplex primer mix gave accurate results in plant samples carrying only one Lr gene, as described above. Our future experiments will be directed towards developing a broad-based multiplex PCR method to target additional 12–13 Lr genes.

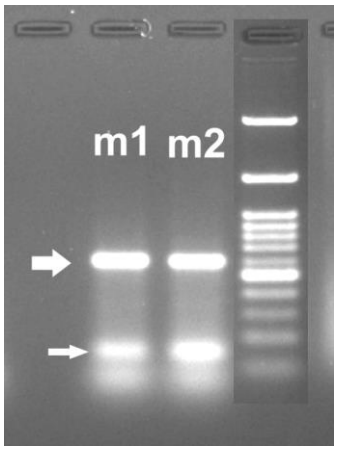

Fig. 6 Bands of two samples (m1, m2), carrying both *Lr28* and *Lr19*, obtained by a PCR run with the multiplex primer mix; the lower arrow and upper arrows indicate *Lr19* and *Lr28*, respectively. Ladder: 100 bp.

Table. 1 A comparison of PCR optimisation conditions of the present study with other studies. The references for each study based on a single gene were given in the table.

	Other studies	Present study
Lr9	Denaturation at 95°C for 15 min, 35 extension cycles of 94°C for 1 min, 64 °C for 1 min 30 s and 72 °C for 2 min 30 s (Moullet et al., 2009)	Denaturation at 94 °C for 1 min, 35 extension cycles [(94°C for 30 s, 57–61°C (ideal 61°C for 30 s), and 64–68°C for 2 min] and final extension at 72 °C for 30min.
Lr19	Denaturation at 94°C for 4 min, 40 extension cycles (92°C for 1 min, 60°C for 1 min, and 72°C for 2 min), and final extension at 72°C for 5 min (Bhawar et al., 2011)	Denaturation at 94°C for 1 min, 35 extension cycles [(94°C for 30 s, 57–61°C (ideal 61°C for 30 s), and 64–68°C for 2 min.] and final extension at 72°C for 30 min.
Lr24	Denaturation at 94°C for 3 min, 38 extension cycles (92°C for 1 min, 68°C for 2 min, and 72°C for 2 min) and final extension at 72°C for 5 min (Nocente et al., 2007).	Denaturation at 94°C for 1 min, 35 extension cycles [94°C for 30 s, 57–61°C (ideal 61°C for 30 s), and 64–68°C for 2 min] and final extension at 72°C for 30 min.
Lr28	Denaturation at 95°C for 2 min, 45 extension cycles (94°C for 1 min, 37°C for 1 min and 72°C for 1 min) and final extension at 72°C for 7 min (Cherukuri et al., 2005).	Denaturation at 94°C for 1 min, 35 extension cycles [94 °C for 30 s, 57–61ºC (ideal 61°C for 30 s), and 64–68°C for 2 min] and final extension at 72°C for 30 min.

Conclusion

We showed that four different *Lr* genes could be reliably screened using the multiplex PCR method. We found that all four genes can be amplified successfully with optimum sample volume, primer and DNA concentration, as well as temperature and PCR reaction times if they are the only gene in the sample. In addition, our multiplex PCR experiments run in single tube involving *Lr19*, *Lr28* and *Lr24* yielded successful results. Further studies are required to design new primers and PCR conditions to allow the amplification of *Lr9* combined with other *Lr* genes in a single multiplex PCR reaction. The present results represent a preliminary study towards our aim of fully optimizing this multiplex PCR method for screening these genes.

Kaynaklar

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