American Journal of Plant Sciences, **2011, 2, 683-687**
doi:10.4236/ajps.2011.25082 Published Online November 2011 (http://www.SciRP.org/journal/ajps)

Identification of AFLP Markers Linked to Leaf Rust Resistance Genes Using Near Isogenic Lines of Wheat

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Received August 20th, 2011; revised October 12th, 2011; accepted October 30th, 2011.

ABSTRACT

*The present investigation was undertaken to find molecular markers linked to leaf rust resistance genes, Lr*9 *and Kharchia local mutant KLM*4-3*B. Preliminary AFLP analysis was carried out with different stocks, a survey of primer combinations with different selective nucleotide indicated that for each primer combination, the number of scorable loci ranged from* 34 *to* 123*. Only a limited primer combination used in the set of parental and near isogenic lines showed a high level of polymorphism for AFLP marker. Putative AFLP marker were found to be linked to Lr*9, *Lr*19 *and KLM*4-3*B. The alien genes were readily identified.*

Keywords*: *AFLP, Leaf Rust, Wheat, Lr*9*, Isogenic Lines

1. Introduction

Wheat exceeds every other grain crop in acreage and production and is, therefore, the most important cereal of the world. With the introduction of semi-dwarf, photoinsensitive, fertilizer responsive and the high yielding varieties of wheat, the wheat production in India has increased from 12 million tonnes in 1966 to 85 million tonnes in the recent years. It is imperative to stabilize the wheat production by reducing the losses due to various diseases including leaf rust, stem rust, yellow rust, Karnal bunt etc. Among the diseases, leaf rust caused by *Puccinia recondita* Roberage ex. Desmaz f.sp. *tritici* is one of the most important and devastating foliar diseases of wheat which cause significant yield losses all over the world [1-8]. Breeding for resistance against leaf rust is an economical, efficient and environmentally safe control measure to reduce these losses [9]. Development of disease resistant varieties is one of the most economical methods of control of diseases like leaf rust. However, growing of rust resistant varieties having single gene for resistance results in rapid evolution of virulent biotypes of the pathogen, and thereby makes the resistance gene ineffective and the variety susceptible to rust. One of the ways to develop varieties with durable rust resistance is to pyramid the genes for resistance in a single variety [10]. It is difficult to pyramid two or more disease resistance genes through conventional means, particularly where the resistance genes in question are effective against all the prevalent pathotypes. However, recent advances in molecular biology has made it possible to pyramid several genes in single line using marker assisted selection (MAS) and tagging of genes with molecular markers is pre-requisite for MAS [11].

A number of rust resistance genes, including leaf rust, have been transferred from wild relatives of wheat into cultivated wheats [12,13]. In India, from the analyses of 2630 samples collected from 17 states, one union territory and Nepal from 2005 to 2008, 31 races were identified among which eight were new [14]. Most of which could not be exploited because of extensive linkage drag. One of the leaf rust resistance genes, *Lr*9 transferred from *Aegilops umbellulata* [15] located on chromosome 6BL, has no undesirable effect associated with it [16]. This gene is effective against all the races of leaf rust currently prevalent in northern India. Similarly, another leaf rust resistance gene identified in (Kharchia local mutant KLM4-3B) is also effective against all the prevalent leaf rust pathotypes in northern India.

Keeping this in view the present study was undertaken to identify molecular markers linked with *Lr*9, *Lr*19 and

KLM4-3B as these genes provide resistance against most of the leaf rust pathotypes of the Indian subcontinent.

2. Materials and Methods

2.1. Plant Material

Near-isogenic lines carrying the leaf rust resistance genes *Lr*9, *Lr*19 and the leaf rust resistant gene of KLM4-3B in the background of WL711 developed at the School of Biotechnology were used along with the donor and the recurrent parents for identifying AFLP markers linked to the two genes.

2.2. Genomic DNA Isolation

Approximately 5 g fresh weight of young leaves were harvested from plants grown in the field and DNA was extracted as per the method of Dellaporta [17].

2.3. Amplified Fragment Length Polymorphism (AFLP) Analysis

AFLP analysis was carried out according to procedures of Vos *et al.* (1995) [18] with minor modifications. The genomic DNA was restricted with two enzymes, a 6-base (rare) cutter Pstl and a 4-base (frequent) cutter Mse1 at 37˚C. The Pst1 and Mse1adapters were ligated to the fragment ends; amplifying a subset of Msel-Pstl fragments with primers that match the adapter and contain additional selective nucleotide at the 3' end; and separating the fragments on denaturing polyacrylamide gel (6%). Sequence of adapters and the primers used for AFLP analysis are given in **Table 1**. To achieve selective amplification of a subset of these fragments, 10 cycles of PCR amplification under following parameters. Thirty seconds denaturation at 94˚C, thirty seconds primer annealing at 65˚C and decreasing one degree temperature in every subsequent cycles and one minute primer extention at 72˚C.

2.4. Separation of Amplified Fragments on Denaturing Polyacrylamide Gel

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 20 min. 1.0 _L 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 50˚C using a Li-Cor DNA Gene Readir 4200 (MWG Biotech. Ebersberg/Germany).

3. Results and Discussion

AFLP Analysis

Preliminary AFLP analysis was carried out with different stocks including recurrent parent WL711, Thatcher + *Lr*9 and *LrKLM*4-3*B* and isogenic lines *i.e.* WL 711 + *Lr*9 and WL 711 + *Lr* KLM4-3B. KLM4-3B, along with these stocks, analysis of HD 2329, Agatha (Lrl9) and isogenic line WL 711. A survey of primer combinations with different selective nucleotide indicated that for each primer combination, the number of scorable loci ranged from 34 to 123 (**Table 2**). Total number of marker loci scored with different primer combination were 682 for WL 711, 629 for HD 2329, 611 for Thatcher+*Lr*9, 515 for Agatha (*Lr*19), 582 for *Lr* KLM4-3B, 629 for WL 711 + *Lr*9, 516 for WL 711 + *Lr*19 and 599 for WL 711 + *Lr* KLM4-3B (**Table 2**). So, minimum number of marker loci (515) amplified were from Agatha (*Lr*19) stock and

Table 1. Sequence of the adapters and primers used for pre-amplification and selective amplification.

Purpose	Oligonucleotide sequences
Adapters	
Pstl-Adapter-Sequence	5' -CTCGTAGACTGCGTACATGCA-3' 3' -CATCTGACGCATGT-5'
Msel-Adapter-Sequence	5' -GACGATGAGTCCTGAG- 3' 3' -TACTCAGGACTCAT-5'
Primers for preamplification	
Pstl-primer	5' -GACTGCGTACATGCAGA-3'
Msel-primer	5' -GATGAGTCCTGAGTAAC-3'
Primers for selective amplification	
Pstl-primer + ACT	5' -GACTGCGTACATGCAGACT- 3'
Pstl-primer + ACC	5' -GACTGCGTACATGCAGACC-3'
Msel-primer + CAA	5' -GATGAGTCCTGAGTAACAA-3'
Msel-primer + CTA	5' -GATGAGTCCTGAGTAACTA-3'
Msel primer + CTG	5' -GATGAGTCCTGAGTAACTG-3'
Msel primer + CTT	5' -GATGAGTCCTGAGTAACTT-3'

Table 2. Number of AFLP loci scored in different stocks using different primer combinations.

Primer combination	*T. aestivum* WL 711	*T. aestivum* HD 2329	Thatcher + *Lr*9	Agatha (*Lr*19)	KML 4-3B	WL711 + *Lr*9	WL711 + *Lr*19	WL711 + *Lr*KLM4-3B
Pst1 + ACC/Mse1 + CTB	110	98	107	70	104	111	78	110
Pst1 + ACC/Mse1 + CTA	119	123	98	98	121	100	103	108
Pst1 + ACC/Mse1 + CAA	98	51	96	34	49	97	40	50
Pst1 + ACC/Mse1 + CTG	67	67	43	59	52	49	49	63
Pst1 + ACC/Mse1 + CTA	79	82	75	94	71	79	89	77
Pst1 + ACC/Mse1 + CTT	108	110	92	84	91	94	82	85
Pst1 + ACC/Mse1 + CTA	101	98	100	76	94	99	75	96
Total number of loci	**682**	**629**	**611**	**515**	**582**	**629**	**616**	**599**

maximum (682) in case of WL 711.

Number of conserved sequence markers were scored separately for different primer combination and it was found that primer Pstl + ACC/Mse1 +CTG amplified 35 sequences common to all the used, whereas Pstl + ACC/ Mse1 + CTG amplified 56, Pstl + ACT/Mse1 + CAA amplified 24, Pstl + ACT/Mse1 + CTG amplified 26 and Pstl + *ACTI* Msel + CTA amplified 32 common sequences. Out of 110 scorable markers (**Figure 1**) amplified by Pstl + ACC/Mse1 + CTG, only one marker (**Figure 1(a)**) was found to be specific in *Lr*KLM4-3B and WL 711 + *Lr*KLM4-3B and were not amplified in any of the other stocks. Further, it was seen that primer combination Pstl + ACT/Msel + CTT amplified three markers (**Figures 2(b)-(d)**) which were specifically amplified in Agatha (*Lr*19) and in isogenic line, WL 711 + *Lr*19 and were not amplified in any of the other stocks (**Figure 2**).Only a limited primer combination used in the set of parental and near isogenic lines showed a high level of polymorphism for AFLP marker as compared to RAPD [19]. Putative AFLP marker linked to *Lr*9 and *Lr*19, the alien genes were readily identified. These primer combinations need to be tried on the relevant F_2 population or RILs for estimation of extent of association before development of STS primers for MAS.

This technique was utilized to clone and map variety specific rice genomic DNA sequence [20]. Many other workers has used this technique in the past for detecting polymorphism, DNA fingerprinting, molecular typing [21,22], genome mapping [19,23], gene tagging [24], genetic diversity analysis [25] and gene expression analysis [26]. AFLP technique for classification of rice germplasm by fingerprinting cytoplasmic male sterile lines of rice was performed and found that the banding pattern of AFLP markers were remarkably consistent [27]. The duplicated CMS lines shared every AFLP band and were thus confirmed as identical genotypes. Thus AFLP analysis conducted in the present study were found to be useful tools in identification of putatively linked markers

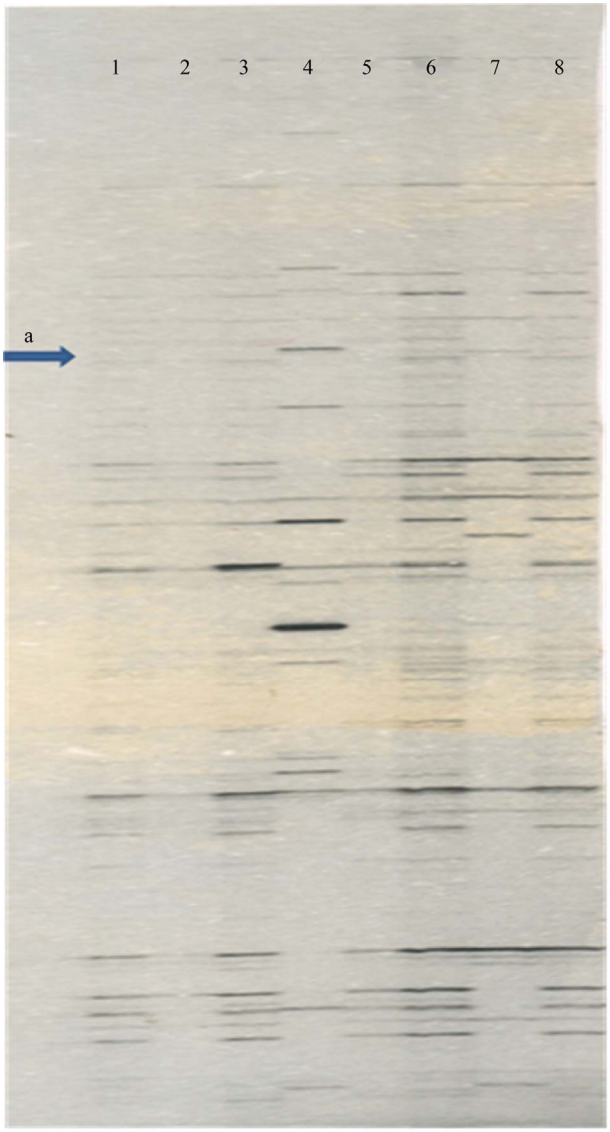

Figure 1. AFLP markers amplified by primer combination Pst1 + ACC/Mse1 + CTG. Lanes 1-8: WL711, HD2329, Thatcher +*Lr*9, *LrKLM*4-3B, Agatha (*Lr*19), WL711 + *Lr*9, WL711 + *Lr*KLM4-3B, WL711 + *Lr*19.

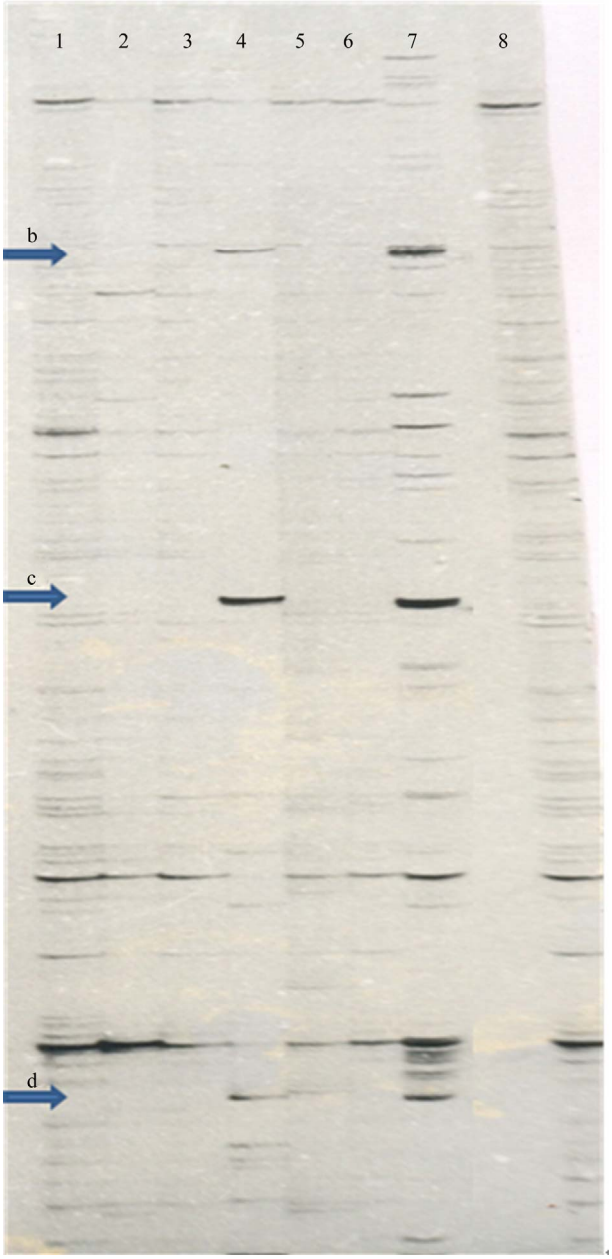

Figure 2. AFLP markers amplified by primer combination Pst1 + ACT/Mse1 + CTT. Lanes 1-8: WL711, HD2329, Thatcher +*Lr*9, Agatha (*Lr*19), *LrKLM*4-3*B*, WL711 + *Lr*9, WL711 + *Lr*19, WL711 + *Lr*KLM4-3B.

to different leaf rust resistant genes. This high reproducebility, rapid generation and high frequency of identifiable AFLP polymorphic bands makes AFLP analysis an attractive approach for molecular analysis in different organisms.

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