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Identification of SSR Marker Linked to Leaf Rust Resistant Gene *Lr24* and Marker Assisted Transfer of Leaf Rust Resistance Genes into Bread Wheat

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ABSTRACT

A study was conducted to identify SSR markers linked to leaf rust resistance genes *Lr24* and *Lr28* and to be used for Marker-Assisted Selection (MAS) to transfer both genes to a widely cultivated wheat variety MP 3299 under rainfed condition. F2 individuals of the cross MP 3299×NIL PBW 343 were used for generating genotypic data employing closely linked SCAR markers S73719 and S421570 to *Lr24* and *Lr*28, respectively, and further subjected to bulk segregant analysis. A total of 70 SSR markers that amplify sequences on long arm of chromosome 3D and long arm of chromosome 4A were used for polymorphism assay between the parents MP 3299 and NIL PBW 343. Eighteen SSRs were polymorphic between the parents, of which 10 were located on chromosome 3DL and eight on chromosome 4AL. Three SSR markers out of 18 polymorphic markers differentiated two contrasting bulks and further used for F2 genotyping. Finally, one SSR marker i.e. 'barc 71' linked to SCAR marker SCS73719 at a distance of 3.36 cM based on the per cent recombination frequency was identified. Thus, the newly identified SSR marker barc 71 linked to *Lr24* can serve as a useful marker in gene pyramiding instead of SCAR marker SCS73719.

Keywords: Bulked segregant analysis, Major gene, Marker-assisted selection, Seedling resistance, Polymorphism.

INTRODUCTION

Wheat (*Triticum aestivum* L. em Thell) is the main staple food in more than 40 countries for over 35 per cent of the world's population. *T. aestivum* is an allohexaploid (2n= 42) with a large genome of 16 giga bases per haploid cell (Bennett and Smith, 1976). In India, wheat is grown over an area of 29.90 million hectare, with a production of 93.9 million tonnes with an average productivity of 3,140 kg per hectare (Anon., 2012). Among various pathogens afflicting wheat, rust diseases have been a major concern and problem for breeders and farmers. Wheat rusts have been reported as devastating, having the ability to destroy the entire susceptible wheat crops in a span of weeks, resulting in large economic losses (Marsalis and Goldberg, 2006). Among three rusts, wheat leaf rust, also known as brown rust, is a serious fungal disease affecting wheat caused by *Puccinia triticina* Eriks.

Leaf rust has the potential to cause yield losses up to 40 per cent (Singh *et al.*, 2002). Development of genetic resistance to rust is the most efficient, cost-effective and environment- friendly approach to prevent the losses caused by rust epidemics. Till date, more than 70 leaf rust resistance genes have been identified in wheat and related species (McIntosh *et al.*, 2013). However, virulent leaf rust resistance races develop very quickly in response to the widespread use of wheat

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cultivars with race specific resistance genes. As the *P. triticina* Eriks population is very large, it would be expected that random mutations and sexual recombination occur in a large number to produce new virulent races. Hence, there are constant efforts in search of the novel resistance genes in order to cope up with the dynamic and rapidly evolving pathogen populations.The use of cultivars with single-gene resistance permits the selection of mutations at a single locus to render the resistance effective in a relatively short time. However, due to selection pressure and evolution, new virulent races of the fungus appear, which increase the need to develop durable resistance. Hence, the use of combinations of genes has been suggested as the best method for genetic control of leaf rust (Roelfs, 1988). This activity of combining resistance genes can be achieved by pyramiding effective resistance genes, but it is difficult to identify the plants in the field for expression of individual resistance genes against the background of other resistance genes. With the advent of molecular marker technology, it is now possible to tackle such complex problems. Application of molecular techniques and Marker-Assisted Selection (MAS) in a breeding program can assist to reach a breeding objective in a shorter period of time. These markers can help in pyramiding resistance genes into single cultivar for durable resistance in segregating populations. Alien leaf rust resistance genes, *Lr24* derived from *Agropyron elongatum* and *Lr28* originating from *Aegilops speltoides* provide effective resistance against all the Indian leaf rust pathotypes. Both *Lr24* and *Lr28* genes exhibited seedling and adult plant resistance (Sohail *et al*., 2014). Chhuneja *et al.* (2011) reported that the combination of *Lr24* and *Lr28* genes provide very high degree of resistances to leaf rust. Hence, the present study was conducted to identify new molecular marker to leaf rust resistant genes using bulk segregant analysis and to transfer wheat leaf rust resistance genes *Lr24* and *Lr28* into MP-3299, a highly susceptible bread wheat cultivar suitable for rainfed condition, using Marker-Assisted Selection (MAS).

MATERIALS AND METHODS

Plant Material

The parent NIL PBW-343 pyramided line with two leaf rust resistance genes *Lr24* and *Lr28* which was confirmed with the help of already reported SCAR markers for *Lr24* (Prabhu *et al.*, 2004) at a distance of 6.4 cM (Cherukuri *et al*., 2003) and *Lr28* at a distance of 3.7 cM (Cherukuri *et al.*, 2005) was crossed with the susceptible variety MP-3299 during Rabi (post monsoon season) 2012 at Dr. Sanjaya Rajaram Wheat Research Laboratory, MARS, UAS, Dharwad. F_1 seeds of the cross MP-3299×NIL PBW-343 was sown at the field of Regional Station, Wellington, Nilgiris during summer 2013. Individual plants of F_1s of the cross MP-3299×NIL PBW-343 were confirmed for the presence of *Lr24* and *Lr28* genes using SCAR markers linked to leaf rust resistance genes *Lr24* and *Lr28* (Table 1) and were advanced to F_2 by selfing. During Rabi 2013, 210 F_2 plants were raised in 42 rows of one meter length with the spacing of 23 cm between rows and 20 cm between plants within a row.

Table 1. Details of SCAR markers linked to the targeted rust resistance genes in the study.

Gene	Marker name	Sequence (5' to 3')	Amplicon size (bp)	Reference
Lr24	$SCS73_{719}$	*TCG TCC AGA TCA GAA TGT G CTC GTCGATTAGCAGTGAG*	719	Prabhu *et al.* (2004)
Lr28	$SCS421_{570}$	*ACA AGG TAA GTC TCC AAC CA AGT CGA CCG AGA TTT TAA CC*	570	Cherukuri *et al.* (2005)

Parental Polymorphism

Parental polymorphism survey was conducted with 70 chromosome arm specific markers comprising of 35 SSRs each from long arm of 3D chromosome and long arm of 4A chromosome (Roder *et al.*, 1998; Guyomarch *et al.,* 2002; Somers *et al.,* 2004; Sourdille *et al.,* 2004; Gupta *et al.,* 2002).

DNA Extraction and PCR Protocol

Total cellular DNA of parents was isolated by using Cetyl Trimethyle Ammonium Bromide (CTAB) method (Dellaporta *et al.,* 1983). PCR reactions were carried out from the purified genomic DNA with the total reaction mixture of 25 µL consisting of 2.5 µL of 40 ηg genomic DNA, 5 µL of 10X Taq buffer (Sigma-Aldrich), 1 µL of 10 mM $MgCl_2$, 1 µL of 2.5 mM dNTPs, 0.5 µL each of forward and reverse primers (5 pmoles), 0.25 µL of 3U Taq DNA polymerase and 12.75 µL sterile distilled water with an overlaid drop of mineral oil. Total reaction mixture was subjected to PCR (Biorad Mastercycler) amplification with the cycling parameters of: 94°C for 2 minutes followed by 94°C for 1 minute, 58°C for 45 seconds, 72°C for 60 seconds for 35 cycles and final extension at 72°C for 10 minutes. PCR products were visualized on 4% superfine agarose (Sigma) gel stained with Ethidium Bromide and ran in 1X TBE buffer at a constant voltage of 90 Volts for two hours and documented in gel documentation system (BioRad XR, Biorad, USA). Low range ruler with a ladder range of 100-3,000 bp was used as a standard molecular marker with known weights.

Bulked Segregant Analysis

The F_2 plants were confirmed for the presence of the two leaf rust resistance genes *Lr24* and *Lr28* using linked SCAR markers. Bulk segregant analysis (Michelmore *et al.*, 1991) was carried out with polymorphic SSR obtained from screening of the two parental genotypes. The F_2 individuals of the cross MP-3299×NIL PBW-343 were used for bulked segregant analysis. Resistant and susceptible DNA bulks were prepared from F_2 individuals by pooling aliquots containing equal amount of DNA (20 ng μL^{-1}) from each of the ten resistant and ten susceptible F_2 individuals based on the presence and absence of the leaf rust resistance genes *Lr24* and *Lr28*. The putative linked SSR markers from bulked segregant analysis were used for the analysis of 210 F_2 individuals of the cross MP-3299×NIL PBW-343 segregating for leaf rust resistance genes. The F_2 plants were scored for SSR markers as 1: P1 Parental type, 3: P2 Parental type and 2: Heterozygote. *Chi*-square test was performed to examine the goodness of fit between the expected Mendelian ratios for the segregation data of the SSR markers analyzed and the association between putatively linked SSR markers and the leaf rust resistance genes in the population using the following formula:

Percent recombination= (Number of recombinants/Total number of samples)×100

Marker Assisted Selection for Leaf Rust Resistance Genes *Lr24* and *Lr28*

Two hundred and ten individual F_2 plants from the cross MP-3299×NIL PBW-343 were analyzed for the presence of both *Lr24*

Table 2. SSR primers used for molecular analysis of leaf rust resistance genes *Lr24* and *Lr28.*

Primer details	*Lr24*	*Lr28*
Total number of primers screened	35	35
Total number of primers amplified	24	20
Polymorphic markers between NIL PBW-343 and MP-3299	10	8
Primer linked to the SCAR marker	1	None

and *Lr28* genes using SCAR markers linked to leaf rust resistance genes *Lr24* and *Lr28*. The plants bearing both genes were subjected to background selection using five polymorphic SSR markers each from long arm of 3D chromosome and long arm of 4A chromosome. The plants with MP-3299 banding pattern were advanced to F_3.

RESULTS AND DISCUSSION

MP-3299 is one of the high yielding early maturing genotype adapted to rain-fed condition in Karnataka; however, it is highly susceptibility to leaf rust disease. This necessitated the incorporation of leaf rust resistant genes into MP-3299. Near isogenic line PBW-343 pyramided with *Lr24* and *Lr28* shows resistance towards the prevailing races of the region was selected as donor for the leaf rust resistance genes for the genetic enhancement of MP-3299 for disease resistance. Already reported SCAR markers *viz.*, $SCS73_{719}$ for *Lr24* (Prabhu *et al.*, 2004) and $SCS421_{570}$ for *Lr28* (Cherukuri *et al.*, 2005) leaf rust resistant genes, were used. These SCAR markers are dominant in nature and, hence, an attempt was made to identify co-dominant SSR marker with high reproducibility which can be used for selecting of homozygous plants in the segregating generations.

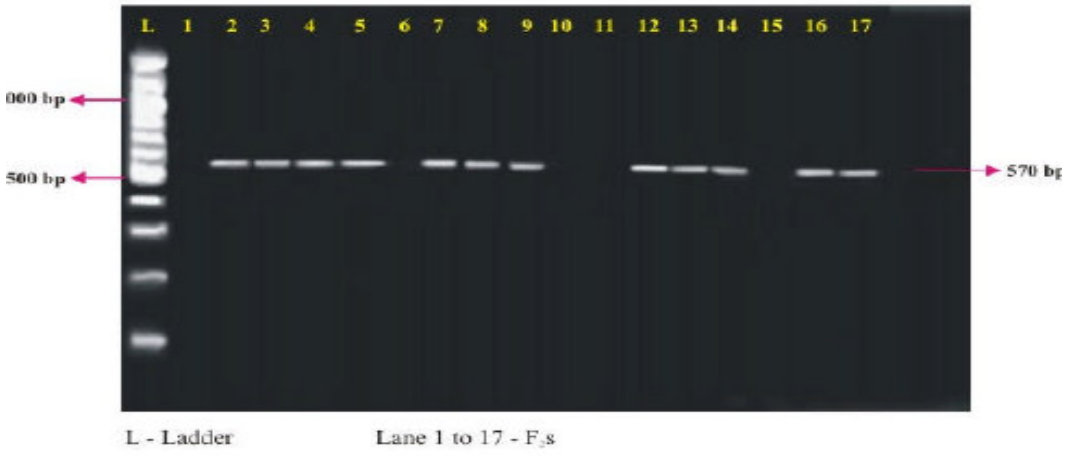
In the donor parent NIL PBW-343, both *Lr24* and *Lr28* genes were successfully confirmed with the help of two SCAR markers $SCS73_{719}$ and $SCS421_{570}$, respectively. These markers have already been utilized by Revathi *et al.* (2010) and Chhuneja *et al.* (2005) in marker assisted selection.The F_1s of the cross MP-3299×NILPBW-343 were confirmed for the presence of both *Lr24* and *Lr28* genes using SCAR markers. The results are in accordance with the earlier findings of Gupta *et al.* (2006) for *Lr24* and Cherukuri *et al.* (2005) for *Lr28*. Molecularly confirmed F_1 plants were selfed to produce 210 F_2 plants.

Out of 70 SSR markers selected based on the location of resistance genes *Lr24* and *Lr28* on long arm of 3D and long arm of 4A chromosomes, respectively, 10 polymorphic markers representing long arm of 3D chromosome and eight representing long arm of 4A chromosome were selected (Table 2). These 18 SSR markers polymorphic between the parents were subjected to bulk segregant analysis (Michelmore *et al.,* 1991) in the F_2 population of the cross MP-3299×NIL PBW-343.Bulks were made based on the genotyping of F_2 segregating population for both leaf rust resistance genes *Lr24* and *Lr28* to identify putative markers linked to these genes. Three SSR markers polymorphic in the parents were also polymorphic between resistant and susceptible bulks, indicating that these markers are putatively linked to leaf rust resistance genes in wheat. These findings are in accordance with the earlier reports employing bulked segregant analysis to identify SSR markers putatively linked to *Pm2* gene (Qiu *et al.,* 2006) and adult plant resistance gene *Lr48* located on long arm of 2B chromosome in wheat (Singh *et al.,* 2011).

The three SSR markers which differentiated the two contrasting bulks were tried on 210 F_2 individuals. Finally, one SSR marker (barc 71) which showed the typical Mendelian segregation ratio of 1:2:1 appeared to be linked with the SCAR marker $SCS73_{719}$ at a distance of 3.36 cM based on the percent recombination frequency. This marker linked to leaf rust resistance gene *Lr24* would be useful in pyramiding the leaf rust resistant gene to susceptible and promising genotypes for yield through marker-assisted selection.

Out of 210 F_2 plants, 108 showed the presence of both *Lr24* and *Lr28* genes based on two SCAR markers (Figures 1 a-b). The results indicated the independent segregation of *Lr24* and *Lr28* genes in the ratio of 9:3:3:1 in F_2 (Table 3). It is essentially due to the fact that *Lr24* is present on long arm of 3D chromosome and

(a)



(b)

Figure 1. Genotyping of F_2 population of the cross MP-3299×NIL PBW-343 using SCAR marker SCS73719 linked to *Lr24* gene. (a) Lane no. 16 is NIL PBW-343 and Lane no. 17 is MP 3299. (b) Lane no. 1 is MP 3299 and Lane no. 2 is NIL PBW-343.

Table 3. Segregation of F2 generation for two leaf rust resistance genes in the cross NIL PBW-343×MP-3299.

Sl No	Number of plants	Genotypic class		$\chi 2^a$
		Observed	Expected	
1	Plants with *Lr24* and *Lr28*	108	118	0.87
2	Plants with only *Lr24*	43	39.375	0.33
3	Plants with only *Lr28*	44	39.375	0.54
4	Plants without *Lr24* and *Lr28*	15	13.125	0.27
	Total	210	210	Calculated $\chi 2$ value 2.01

[a] Table $\chi 2$ value at 3 degrees of freedom= 2.60.

Lr28 is present on long arm of 4A chromosome. Similar kind of segregation for leaf rust resistance genes in the ratio of 9:3:3:1 was reported by Kaur *et al.* (2012). These 108 F_2 plants were subjected to selection with five polymorphic SSR markers each on long arm of 3D chromosome and long arm of 4A chromosome, which resulted in the identification of 24 plants genetically similar to MP-3299.This kind of successful marker-assisted selection programs for leaf rust resistant genes were reported earlier (Singh *et al*., 2004; Chhuneja *et al*., 2011). These plants were selfed and advanced to F_3 generation for isolation of progenies similar to MP-3299 with both resistance genes present through genotyping and phenotyping.

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شناسایی نشانگر SSR مربوط به ژن *Lr24* مقاوت به زنگ برگ و انتقال ژن های مقاومت به زنگ برگ به گندم نان به کمک نشانگر

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چکیده

هدف این پژوهش شناسایی نشانگر های SSRمربوط به ژن های مقاومت به زنگ برگ شامل *Lr* 24و *Lr* 28 بود برای استفاده در گزینش به کمک نشانگر (MAS) به منظور انتقال هر دو ژن به یک کولتیوار گندم به نام MP 3299که به طور گسترده ای در مناطق دیمکاری کشت می شود. به این منظور، افراد نسل ۲ (F2) تلاقی MP 3299 x NIL PBW 343 برای تولید داده های

ژنوتیپیکی با کار برد نشانگر های SCAR S73719و S421570 نزدیک به هم و به ترتیب مربوط به *Lr* 24و *Lr* 28استفاده شدند و تحت تجزیه مخلوط تفرق یافته ها (bulk segregant analysis) قرار داده شدند. در کل، ۷۰ نشانگر SSRکه توالی روی بازوی بلند کروموزوم Dو بازوی بلند کروموزوم A4 را تکثیر می کنند برای آزمون چند شکلی بین والدها شامل MP 3299و NIL PBW 343 به کار گرفته شد. هیجده SSRدر بین والدها چند شکلی بودند که ۱۰ تای آنها روی کروموزوم 3DLو ۸ عدد روی کروموزوم 4AL قرار داشتند. از هیجده نشانگر چند شکلی، سه نشانگر SSRدو مجموعه متمایز و متفاوت (differentiated two contrasting bulks) داشتند و برای بررسی ژنوتیپ نسل دوم استفاده شدند. بالاخره، یک نشانگر SSRیعنی "barc 71" که به نشانگر CS73719 مربوط می شد در فاصله cM ۳/۳۶ بر مبنای درصد بسامد نو ترکیبی (recombination frequency) شناسایی شد. به این قرار، می توان به جای نشانگر SCAR به نام SCS73719، از نشانگر SSR تازه شناسایی شده به نام barc 71 که *به Lr24*مربوط است به عنوان نشانگر موثری در هرمی کردن ژن (gene pyramiding) بهره جست.