Identification of SSR Marker Linked to Leaf Rust Resistant Gene \textit{Lr24} and Marker Assisted Transfer of Leaf Rust Resistance Genes into Bread Wheat

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\textbf{ABSTRACT}

A study was conducted to identify SSR markers linked to leaf rust resistance genes \textit{Lr24} and \textit{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. F\textsubscript{2} individuals of the cross MP 3299\times\text{NIL PBW 343} were used for generating genotypic data employing closely linked SCAR markers S73719 and S421570 to \textit{Lr24} and \textit{Lr28}, 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 \text{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 F\textsubscript{2} 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 \textit{Lr24} can serve as a useful marker in gene pyramiding instead of SCAR marker SCS73719.

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

\textbf{INTRODUCTION}

Wheat (\textit{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. \textit{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 hectares, 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 \textit{Puccinia triticina} Eriks.

Leaf rust has the potential to cause yield losses up to 40 per cent (Singh \textit{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 \textit{et al.}, 2013). However, virulent leaf rust resistance races develop very quickly in response to the widespread use of wheat.
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. F₁ 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 F₁ 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 F₂ by selfing. During Rabi 2013, 210 F₂ 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Marker name</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Lr24 | SCS73₇₇₉    | TCG TCC AGA TCA GAA TGT G  
CTC GTCGATTAGCAGTGGAG |
| Lr28 | SCS421₅₇₀  | ACA AGG TAA GTC TCC AAC CA  
AGT CGA CCG AGA TTT TAA CC |

Prabhu et al. (2004)  
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 Trimethyl 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 ng genomic DNA, 5 µL of 10X Taq buffer (Sigma-Aldrich), 1 µL of 10 mM MgCl₂, 1 µL of 2.5 mM dNTPs, 0.5 µL each of forward and reverse primers (5 pmol), 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.

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

<table>
<thead>
<tr>
<th>Primer details</th>
<th>Lr24</th>
<th>Lr28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of primers screened</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Total number of primers amplified</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Polymorphic markers between NIL PBW-343 and MP-3299</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Primer linked to the SCAR marker</td>
<td>1</td>
<td>None</td>
</tr>
</tbody>
</table>

Bulked Segregant Analysis

The F₂ 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 F₂ individuals of the cross MP-3299×NIL PBW-343 were used for bulked segregant analysis. Resistant and susceptible DNA bulks were prepared from F₂ individuals by pooling aliquots containing equal amount of DNA (20 ng µL⁻¹) from each of the ten resistant and ten susceptible F₂ 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 F₂ individuals of the cross MP-3299×NIL PBW-343 segregating for leaf rust resistance genes. The F₂ 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 F₂ plants from the cross MP-3299×NIL PBW-343 were analyzed for the presence of both Lr24
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 F3.

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 F1_{8} of the cross MP-3299xNILPBW-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 F1 plants were selfed to produce 210 F2 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 F2 population of the cross MP-3299xNIL PBW-343. Bulks were made based on the genotyping of F2 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 F2 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 F2 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 F2 (Table 3). It is essentially due to the fact that Lr24 is present on long arm of 3D chromosome and
Figure 1. Genotyping of F2 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.

<table>
<thead>
<tr>
<th>Sl No</th>
<th>Number of plants</th>
<th>Genotypic class</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>1</td>
<td>Plants with Lr24 and Lr28</td>
<td>108</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>Plants with only Lr24</td>
<td>43</td>
<td>39.375</td>
</tr>
<tr>
<td>3</td>
<td>Plants with only Lr28</td>
<td>44</td>
<td>39.375</td>
</tr>
<tr>
<td>4</td>
<td>Plants without Lr24 and Lr28</td>
<td>15</td>
<td>13.125</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>210</td>
<td>210</td>
</tr>
</tbody>
</table>

$^a$ 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 F2 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 F3 generation for isolation of progenies similar to MP-3299 with both resistance genes present through genotyping and phenotyping.

REFERENCES

زنوتیپیکی با کار برد نشانگر های Lr24 و Lr28 به 24 و 28 بهبود می‌یافت. استفاده شدند و تحت تجزیه مخلوط تفرق یافته‌ها (analysis) قرار داده شدند. در کل، 70 نشانگر SSR پایالی روي بازوي بلند کروموزوم D که تاکید می کند برای آزمون دوگانه شامل 3299 و MP بازوي بلند کروموزوم A4 را تکرار می کند. برای آزمون دوگانه شامل 10 تای آنها به کار گرفته شد. هجده بردار سر از کروموزوم السیزک ده نشانگر در دو روش داشتند. از هجده نشانگر چند پایگاه متمایز متشکل از(cM) داشتند (differentiated two contrasting bulks متنوا (mapping) نشانگر SSR و برای نوع زنوتیپیک نسل دوم استفاده شدند. بالاخره، یک نشانگر با نام "barc 71" یعنی SSR "sbarc19" که به نشانگر CS 366 cM مربوط می‌شد در فاصله LS3719 به تکمیل شناسایی شد. به این قرار، می توان به جای نشانگر به نام SCAR "sCS73719" تا نشانگر SSR "sCS73719" و "sCS73719" به عنوان Lr24 "sbarc 71" نشانگر مولتی در هر می کردن زنوتیپ به پره جست. (gene pyramiding)