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Validation and incorporation of leaf rust resistance genes *Lr9*, *Lr19*, *Lr24* and *Lr26* through molecular markers in wheat (*Triticum aestivum* L.)

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Abstract

Molecular markers for the leaf rust resistance genes *Lr9*, *Lr19*, *Lr24*, rye specific chromatin (1BL.1RS translocation, *Lr26*), and *Lr28* were validated in the parental lines of wheat (*Triticum aestivum* L.). The markers for *Lr9*, *Lr19* and *Lr26* were able to discriminate the lines with specific genes/chromatin from the lines that did not carry these genes. The *Lr24* marker showed polymorphism between positive and negative controls but in white grained genotypes the amplified band was faint and inconsistent as compared to red seeded lines. The marker used for detecting *Lr28* was not polymorphic among the tested carrier and non-carrier lines. The markers of *Lr19* and *Lr24* were utilized for authenticating the presence of specific genes in the advanced breeding lines whereas rye-chromatin specific marker was used for marker assisted selection of *Lr26*. Genetic stocks with combined resistance genes *Lr19* + *Lr26* + *Sr25* + *Sr31* + *Yr9* + *Yr27* and *Lr24* + *Lr26* + *Sr24* + *Sr31* + *Yr9* + *Yr27* were developed in the background of PBW 343 with the aid of pedigree information, host-pathogen interaction and molecular marker.

Key words: Wheat, leaf rust resistance, molecular markers, gene pyramiding, marker validation

Introduction

Leaf rust (*Puccinia triticina* Eriks.) occurs through out the wheat growing regions of our country and it is one of the most important foliar diseases of wheat (*Triticum aestivum* L.) in India. Cultivation of rust resistant varieties is the economical and eco-friendly method of disease control. The resistance genes incorporated in the cultivars are mostly short lived because of the continuous evolution of new pathotypes [1]. In order to alleviate the problem of frequent breakdown of resistance genes several strategies have been suggested [2, 3]. One of the effective remedy for increasing the longevity of resistance genes is to develop varieties with more than one effective resistance gene. Through conventional techniques it is not possible to confirm the incorporation of two or more rust resistance genes in the absence of differential host pathogen interaction. However, with the aid of molecular markers it is possible to pyramid more than one resistance genes. Though several leaf rust resistance genes have been tagged with DNA markers in the last decade [4-9], it is of least importance for gene pyramiding unless the validity of such molecular markers is tested in the targeted donor and recipient parents which must show distinct polymorphism. It, therefore, stands reason to validate the available molecular markers for the genes to be pyramided. As an out come of pre-breeding for rust resistance, segregating populations with multiple gene combinations were ready for the application of marker assisted selection. In the present report, molecular markers reported for *Lr9*, *Lr19*, *Lr24*, *Lr26* and *Lr28* were validated in the parents and thereafter genetic stocks with combined resistance for *Lr19* + *Lr26* and *Lr24* + *Lr26* were developed through combined efforts of Host Pathogen Interaction and molecular markers.

Materials and methods

Plant material for molecular marker validation: Seven lines carrying *Lr9 viz.*, *CS* + *Lr9*, HP 1633 and 5 lines of the cross PBW343/HP1633; eight lines carrying *Lr19 viz.*, *Tc* + *Lr19*, FLW 24, FWW 2 and 5 lines of the cross PBW 343/*Tc* + *Lr19*; ten lines carrying *Lr24 viz.*, Agent, Arkan, Blueboy II, FWW 2, FLW 20 and 4 lines of the cross PBW 343/PH 137 carrying *Lr24*, seven lines with *Lr26 viz.*, WH 542, UP 2338, PBW 343 and 4 lines of the cross PBW 343/Arkan; seven lines carrying *Lr28 viz.*, CS-2DM3/8, UP 2338/CS-2DM3/8, PBW 343/CS-2DM3/8, HUW 234/CS-2DM3/8. Lines devoid of the targeted genes were kept as negative control for each marker.

Molecular markers: Molecular markers of *Lr9* [4], *Lr19* [5], *Lr24* [7], *Lr26* [8] and *Lr28* [9] were validated. Markers for *Lr19* and *Lr24* were utilized for authenticating the incorporation of these resistance genes in the genetic stocks. *Lr26* marker was used for marker assisted selection.

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Plant material for applying marker assisted selection: F_2 population and F_3 lines from the cross PBW 343/*Tc* + *Lr19* and PBW 343/Arkan, were screened at seedling stage for leaf rust resistance as well as *Lr26* marker. The F_1, F_2 and F_3 generations were tested with leaf rust pathotype 21R55 whereas the advanced generations were tested at seedling stage with three pthotypes of leaf rust *viz.*, 121R63-1, 121R127, 21R55; two pathotypes of stem rust *viz.*, 62G29 and 62G29-1 and two patotypes of yellow rust *viz.*, 46S103 and 46S119.

Pathological testing conditions: The F_2 and F_3 seedlings were raised in the aluminum bread pan trays comprising ten rows with the 7^{th} row of each tray as a susceptible check, Agra local. Fully expanded primary leaves were inoculated with uredospores suspended in light weight, non-phytotoxic isoparaffinic oil (soltrol). The inoculated seedlings were kept in a saturated humid glass chamber for 48 hours. The seedlings were then transferred on to the glass house benches at about 22°C. Infection types (IT's) were recorded 14 days after inoculation and were classified according to the standard method [10]. The IT's o; (naught fleck), ; (fleck), ; -(fleck minus), ; 1 (fleck one), 1 (one), 2 (two) and X (mesothetic) were classified as the resistant reactions whereas IT 3 (three) and 3+(three plus) were classified as susceptible reactions.

DNA isolation and PCR amplification: DNA was extracted by CTAB method [11]. Amplifications were performed in PTC-200 Thermal Cycler (MJ Research, Waltham, MA). PCR products of *Lr9*, *Lr24* and *Lr26*, were analyzed in 1.5% agarose; *Lr19* in 3% agarose and *Lr28* in 2% agarose gel in 0.5 × TAB buffer. The PCR conditions for different molecular markers are presented in Table 1.

Selection strategy: The F_2 generated from individual F_1's were tested with pathotype 21R55 which is virulent on *Lr26* but avirulent on *Lr19* and *Lr24* genes. Fifty resistant seedlings were transplanted in the field and DNA was isolated from each of the resistant plants which were subjected to molecular marker analysis for the detection of linked genes *Lr26/Sr31/Yr9*. The plants positive for the molecular markers were selected and advanced to next generation. The F_3 progenies were tested at the seedling stage with leaf rust pathotype 21R55 and the non-segregating families were transplanted in the field. Twelve plants from each of the non-segregating F_3 progenies were tagged randomly and DNA was isolated from the tagged plants for applying marker assisted selection of *Lr26* gene. The F_3 families that did not segregate for molecular marker were advanced to the next generation. Incorporation of specific resistance genes were confirmed in the F_4 generation through progeny testing of 30-40 individuals per family.

Results and discussion

Validation of markers: The 1.1 kb band was specifically amplified in the lines carrying *Lr9* and absent in the negative control lines which have other leaf rust resistance genes *viz.*, *Lr24* and *Lr26* (Fig. 1). Similarly, a 132bp fragment was amplified only in the lines with *Lr19* (Fig. 2). Two independent PCR based markers were earlier reported for *Lr24* [6, 7]. One stem rust resistance gene *Sr24* (completely linked with *Lr24*) molecular marker was recently developed [12]. Among the two published markers of *Lr24*, the marker developed by Dedryver *et al.*, 1996 [7] has been used to test its usefulness in breeding programme. The 700bp marker was specifically amplified in the cultivars known to posses *Lr24* [Fig. 3]. However, the marker was faint as well as inconsistent in the white grained *Lr24* lines. The rye-chromatin specific marker, 1.5kb fragment, was specifically amplified in the lines carrying 1BL.1RS translocation (*Lr26/Sr31/Yr9*) (Fig. 4). The marker for *Lr28*, 378 bp band, was not polymorphic among the carrier and non-carrier lines (Fig. 5).

Though the marker for *Lr24* was specifically amplified in carriers but its amplification was not consistent in the white grain lines. The reason for this anomaly may be attributed to the fact that linkage between the *Lr24* gene and the marker was established in the mapping population derived from parents with red seed *viz.*, RL 6064 and Chinese Spring [7] and

Table 1. PCR profile of molecular markers for the genes *Lr9*, *Lr19*, *Lr24*, *Lr26* and *Lr28* in wheat

Genes	Components	Cycles	Reference
Lr9 (SCAR)	2mMMgCl$_2$, 100µMdNTP, 40η M primer, 0.5U Taq Polymerase, 50ng DNA	1 × 94°C6' 40 × 94°C 1'; 62°C l'; 72°C2'; 1 × 72°C4'	Schachermayr *et al.*, 1994 [4]
Lr19 (SCAR)	2mMMgCl$_2$, 0.2mMdNTP, 12.5p moles primer, 0.6U Taq Polymerase, 50ng DNA	1 × 94°C4'; 30 × 94°C; 0.5';60°C 0.5'; 72°C 0.5' 1 × 72° C5'	Prins *et al.*, 2001 [5]
Lr24 (SCAR)	2mMMgCl$_2$, 200µMdNTP, 0.6µM primer, 1.0U Taq Polymerase, 50ng DNA	1 × 94°C0.5' 38 × 94°C 1.5'; 68°C2'; 72° C2' 1 × 72° C5'	Dedryver *et al.*, 1996 [7]
Rye-Chromatin (BL.1RS)	2mMMgCl$_2$, 0.2mMdNTP, 0.1µM primer, 0.5U Taq Polymerase, 50ng DNA	1 × 94°C0.25' 45 × 94° C 1'; 55°C 2'; 72° C 1' 1 × 72°C5'	Fransis *et al.*, 1995 [8]
Lr28 (SCAR)	2mMMgCl$_2$, 100µMdNTP, 40ηM primer, 1.0U Taq Polymerase, 50ng DNA	1 × 94° C6' 35 × 94° C 1'; 50°C l'; 72° C 2' 1 × 72°C5'	Naik *et al.*, 1998 [9]

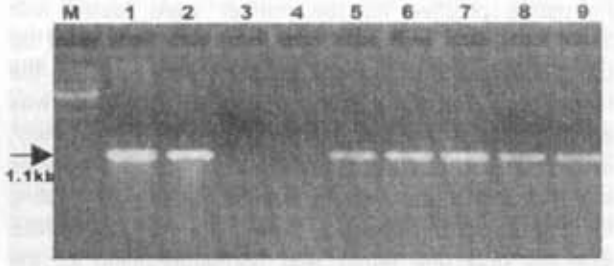

Fig. 1. M-Marker lane, 1:HP1633, 2: CS*Lr9*, 3:PBW 343 (*Lr26*), 4: Agent (*Lr24*), 5-9: Lines of the cross PBW 343/HP1633 (*Lr9*)

Fig. 2. M-Maker lane, 1: *TcLr19*, 2: FLW 24 (*Lr9*), 3: FWW 2 (*Lr19*), 4-8: Lines of the cross PBW 343/*Tc Lr19*, 9: CS*Lr9*, 10: Agent (*Lr25*), 11: PBW 343 (*Lr26*), 12: *Lr29*

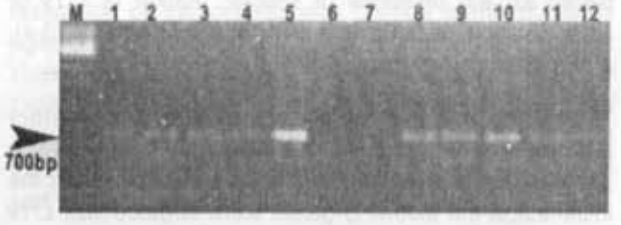

Fig. 3. M-Maker, 1-4: Lines of the cross PBW 343/PH137 carrying *Lr24* (amber seed) 5-Agent (*Lr24* red seed), 6: CS*Lr9*, 7: *TcLr19*, 8: Blueboyll (red seed), 9: PBW 343/Arkan (red seed), 10: Arkan (*Lr24* red seed), 11: FWW2 (*Lr24* amber seed), 12: FLW 20 (*Lr24* amber red)

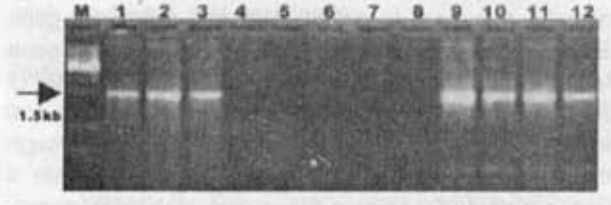

Fig. 4. M-Maker lane, 1: UP 2338 (*Lr26*), 2: PBW 343 (*Lr26*), 3: WH 542 (*Lr26*), 4: Cappelle Desprez, 5: CS*Lr9*, 6: Tc*Lr19*, 7: Tc*Lr32*, 8: Agent (*Lr24*), 9-12: Lines of the cross PBW 343/Arkan

Fig. 5. M-Marker, 1: CS2D2M3/8, 2: PBW 343 + *Lr28*, 3: UP 2338 + *Lr28*, 4: HUW 234 + *Lr28*, 5: Compair (lack *Lr28*), 6-8: Lines with *Lr28* from the cross PBW 343/CS2D2M *Lr28*, 9-11: Lines without *Lr28* from the cross PBW 343/CS2D2M *Lr28*, 12: Tc + *Lr26*

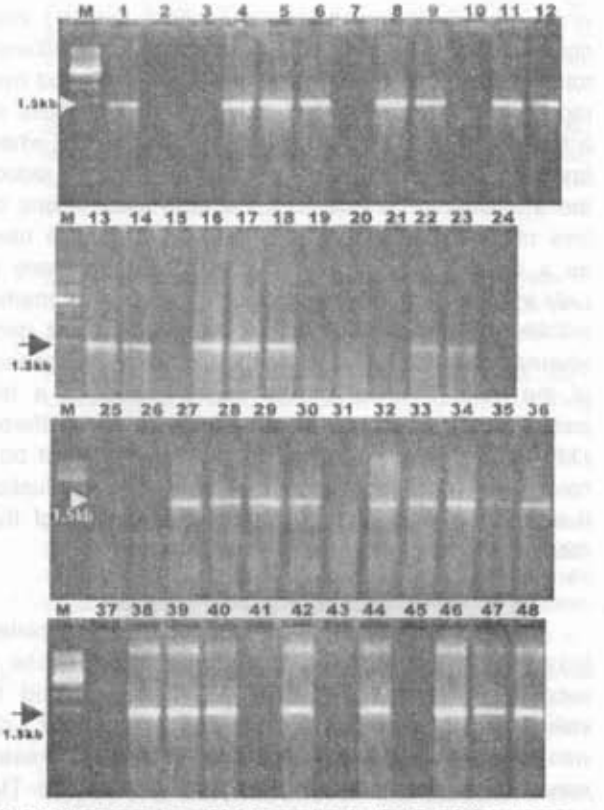

Fig. 6. Segregation of Lr26 marker in the F_2 of the cross PBW 343/Tc*Lr19*

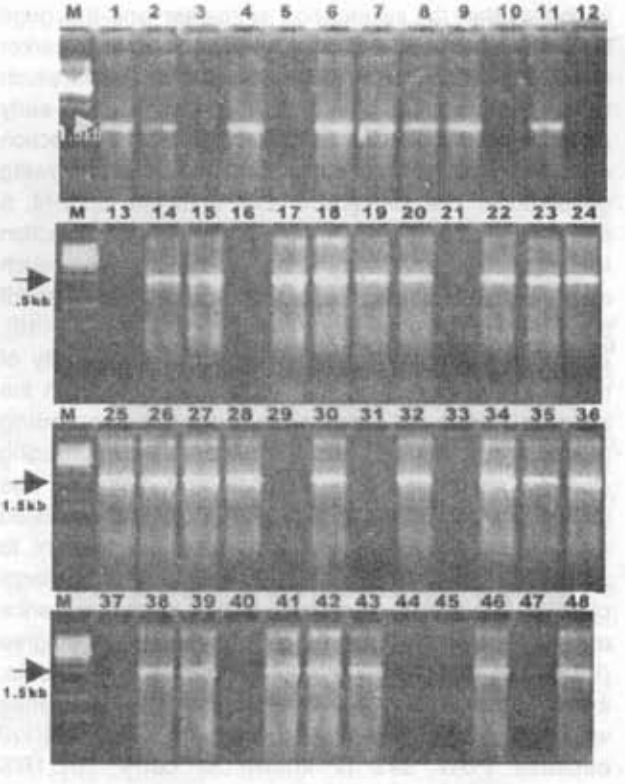

Fig. 7. Segregation of *Lr26* marker in the F_2 of the cross PBW 343/Arkan

white grain *Lr24* genotypes have either reduced alien chromatin or it has chromatin derived from a different translocation [12]. Since white grains are preferred over red grains, this marker has only limited usefulness as a tool for marker assisted selection in the Indian wheat breeding programme because this marker will reduce the efficiency of selection in the early generations by loss of desirable genotypes. However, it can be used as a devise to postulate or authenticate presence of *Lr24* in the advanced lines because presence of marker will be a definite proof of the presence of the gene whereas absence of the marker will indicate the absence of the gene in most of the cases except in a few instance the gene may be actually present. A different *Lr24* marker [7] was utilized for MAS by [13] but both have used red seeded donor of *Lr24* (Arkan, Blueboy II and Thacther + *Lr24*). Therefore, the utility of that marker in white grain lines is not known.

The *Lr28* marker [9] was reported to be completely linked to the gene but its linkage was proved loose in subsequent studies [14, 15]. We have retested its validity in the parents since the donor source of *Lr28* was different in our breeding population. The present results also clearly support the previous studies [14, 15] and hence, the marker for *Lr28*, which is not completely linked to the gene, has no use in MAS. In the context of pyramiding of resistance genes, it is important that the association of marker and the gene is complete so that the lines recovered through marker assisted selection are fixed for resistance genes which would generate the desired gene combinations in early generations and leaving ample scope for the selection of other important agronomic characters in the following generations. The markers for *Lr9*, *Lr19* and *Lr26* [4, 5 and 8] could be utilized in the marker assisted selection for resistance gene pyramiding involving parents such as PBW 343. Similar conclusions were drawn about the utility of *Lr19* and *Lr9* markers in MAS [15, 16]. *Utilisation of marker for selection*: Once the validity of the aforesaid molecular markers were confirmed in the parental lines of the anticipatory pre-breeding programme, in the next phase a comprehensive breeding strategy, as described in materials and methods, was laid down for their application in marker assisted selection. In the F_2, out of fifty seedlings resistant to 21R55, 32 plants of PBW 343/*Tc* + *Lr19* and 33 plants of PBW 343/Arkan were also positive for the presence of rye-chromatin in (Figs. 6-7). On the basis of pedigree (PBW 343/*TcLr19* or PBW 343/Arkan) of the parents, it was obvious that the rye-chromatin in these lines was 1BL.1RS translocation carrying *Lr26/Sr31/Yr9* because PBW 343 is known to carry 1BL.1RS translocation whereas *TcLr19* and Arkan does not carry any rye chromatin. The F_3 progenies derived from the F_2 plants positive for the marker were tested with 21R55 and the non-segregating lines were picked up thus enabling the fixation of *Lr19* and *Lr24* in the respective crosses (Table 2). Marker analysis was applied on the fixed lines for *Lr19* and *Lr24* namely, seven families of PBW 343/*Tc* + *Lr19* and 8 progenies of PBW 343/Arkan. Two F_3 lines were non-segregating for rye-chromatin specific marker in the *Lr19* cross whereas only one family was non-segregating for the other cross involving *Lr24*. None of the F_4 progenies derived from the three selected F_3 families, fixed for both resistance and marker, segregated in a population of 30-40 plants in the seedling resistance test with pathotype 21R55 (virulent on *Lr26* but avirulent on *Lr19* and *Lr24*) which confirmed the fixation of *Lr19/Lr24*. It was absolutely necessary to test the segregation of marker in the F_4 since only 12 plants per F_3 family were tested for marker analysis. The F_4 progenies derived from the three selected F_3 families, fixed for both resistance and marker, did not segregate for marker in a population of 36 individuals. In the F_4 lines, random plants from the respective cross were tested for the presence of specific marker of *Lr19* or *Lr24*. All plants had the requisite band (Fig. 3-4), authenticating presence of *Lr19/Lr24*.

The ultimate objective of the anticipatory pre-breeding programme at Flowerdale is to develop genetic stocks with combined resistance genes. In the initial stage the following genes were targeted *viz.*, *Lr19* + *Lr26* + *Sr25* + *Sr31* + *Yr9* + *Yr27* and *Lr24* + *Lr26* + *Sr24* + *Sr31* + *Yr9* + *Yr* PBW 343. This objective was not attainable solely through conventional techniques because the phenotypic effect of leaf rust resistance gene *Lr26* is masked in presence of *Lr19* or *Lr24*. It is hard to detect stem rust resistance gene *Sr31* in the presence of *Sr25* or unknown *Sr* gene from Arkan which are resistant to all pathotypes of stem rust. Similarly, it is not possible to postulate *Yr9* when it is present along with *Yr27*. However, through the combined efforts of HPI and molecular marker it was possible to combine the above resistance genes. The genes *Sr31* and *Yr9* were incorporated along with *Lr26* because *Lr26/Sr31/Yr9* are completely linked.

The homozygous F_3 line carrying *Lr19* + *Lr26*, selected with the help of HPI and marker, had not lost the *Yr27* gene from PBW 343. Similarly, one of the two homozygous F_3 line carrying *Lr24* + *Lr26*, selected with the help of HPI and marker, was segregating for *Yr27* gene from PBW 343 and *Sr* gene present in Arkan. The F_4 lines resistant to pt. 46S119 of stripe rust and nearly immune to pt. 62G29-1 of stem rust were selected and thus *Yr27* gene from PBW 343 and *Sr* gene from Arkan were also fixed in the later generations. It was possible to recover *Sr* (Arkan) from

one and two F_3 line and *Yr27* from two F_3 lines that were fixed for leaf rust resistance genes *Lr24* + *Lr26* and *Lr19* + *Lr26*, respectively, but this was not enough to recover genotypes that would withstand the high selection pressure and stringent agronomic requirements of the actual breeding programme. It did not affect our objectives since the aim was not to generate breeding lines but to obtain superior genetic stocks with combined resistance that could be used as a parent of the crossing programme. Through the use of molecular markers leaf rust resistance gene *Lr26* was combined with *Lr19* or *Lr24* wherein marker for *Lr26* was used as a selection tool and markers of *Lr19* and *Lr24* assisted in authentication of incorporation of these genes in the final genetic stocks.

Completely linked markers are very useful and convenient tool for selection of rust resistance genes in the early generations. Marker assisted selection is potentially useful for breeding for rust resistance and offers opportunity to select desirable lines on the basis of genotype rather than phenotype, for combining different resistance genes which would enhance the resistance of the cultivar and provide some durability against rust diseases. In the present investigation genetic stocks with combined rust resistance genes (*Lr19* + *Lr26* + *Sr25* + *Sr31* + *Yr9* + *Yr27* and *Lr24* + *Lr26* + *Sr24* + *Sr31* + *Yr9* + *Yr27*) were developed with the aid of pedigree information, host-pathogen interaction and molecular marker.

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