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Marker assisted selection to pyramid seedling resistance gene *Lr24* and adult plant resistance gene *Lr48* for leaf rust resistance in wheat

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Abstract

One *Agropyron elongatum* derived dominant seedling resistance gene (*Lr24*) and one recessive adult plant resistance (APR) gene (*Lr48*) for leaf rust resistance, were pyramided together employing marker assisted selection (MAS) with sequence characterized amplified region (SCAR) marker $SCS1302_{607}$ tagged to the gene *Lr24* and two random amplified polymorphic DNA (RAPD) markers flanking the gene *Lr48*, $S3_{450}$ and $S336_{775}$ in wheat. The RAPD marker $S3_{450}$, linked in repulsion phase amplified a 450 bp marker fragment corresponding to the recessive resistance allele and the other marker $S336_{775}$ linked in coupling phase amplified a 775 bp marker fragment corresponding to the dominant susceptibility allele of the *Lr48* locus. Parental genotypes comprised of the near isogenic line (NIL) of the most widely grown cultivar PBW343 possessing *Lr24* and an Australian cultivar CSP44 possessing APR gene *Lr48*. Phenotyping was done after inoculating with pathotype 77-5 of *Puccinia triticina* and integrated with MAS under controlled conditions of Phytotron (for F_1 and F_3 generations) and natural field conditions (for selection in F_2 and F_4 generations). Agronomic suitability of the marker positive plants was integrated for generating the F_3 families and selecting the F_4 subfamilies. MAS facilitated the identification of 26 F_4 subfamilies fixed for both the genes with desirable plant type from three F_3 families homozygous for dominant gene *Lr24* selected from 38 F_2 single plants homozygous for APR *Lr48*. The selected F_4 subfamilies were furthered for seed bulking and yield performance in F_5 generation.

Key words: Marker assisted selection, adult plant resistance, pyramiding, leaf rust resistance; *Lr48, Lr24*, RAPD, SCAR

Introduction

Leaf rust caused by *Puccinia triticina* Eriks. (Syn: *Puccinia recondita* Roberge ex Desmaz. f. sp. *tritici*) is known to cause damage to wheat in all the wheat cultivated regions in India and is known to remain longest in a season compared to the other two rusts. The impact of leaf rust disease on yield reduction in wheat is well documented globally, which ranges from 10% under moderate to 65% under intense epidemics and depending on the stage of crop when the initial rust infection occurs [1]. As many as 61 *Lr* genes have been reported in wheat and its relatives. While most of the *Lr* genes are operative right from seedling stage, some are operative at adult plant stage showing increasing levels of resistance with the increasing age of the plant (adult plant resistance or APR). Where the adult plant resistance functions only in the adult stages [2], seedling resistance is expressed on first leaf stage and remains effective throughout the growing period [3]. Hence the use of combinations of genes, which could be effective at different stages, irrespective of whether they are major or minor, has been suggested as the best method for genetic control of leaf rust [4]. It was suggested that durable resistance to leaf rust may be obtained by combining seedling specific genes with adult plant resistance. Though this can be achieved by pyramiding effective resistance genes, these are difficult to monitor in the field due to the inability to distinguish the expression of individual resistance genes or due to lack of availability of virulence in the pathogen to differentiate the genes.

With the advent of molecular marker technology it is now possible to tackle such complex problems. DNA-based molecular markers have several advantages over the traditional phenotype based selection especially when disease-escaped-susceptible

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plants are likely to be confused for resistant plants. Further, in the case of APR pyramiding strategy, its detection is not possible in the presence of a seedling resistance gene due to masking effect. In India, the dominant seedling resistance gene *Lr24* derived from *Agropyron elongatum* and the recessive APR gene *Lr48* have been reported to be effective against the pathotypes of the sub-continent [5, 6] and molecular markers linked to gene *Lr24* and *Lr48* have been identified [6, 7]. This paper reports an attempt undertaken to pyramid the combination of the genes *Lr24* and *Lr48* together in an agronomically desirable wheat background by employing molecular marker assisted selection (MAS).

Material and methods

Plant material

The material used in the current study included CSP44, a selection from the Australian cultivar, Condor, carrying a recessive APR gene (*Lr48*) obtained from Prof. R. G. Saini of Punjab Agricultural University, Ludhiana, India and a near isogenic line carrying the gene *Lr24* in the background of the most cultivated high yielding Indian variety of wheat, PBW343 (referred to as PBW343 + Lr24) developed in the National Agricultural Technology Project by Punjab Agricultural University, Ludhiana both of which were used as resistant parents. To facilitate phenotyping for disease reaction, a local wheat variety Agra Local was used as a control and infector to spread the disease in the field. The material was advanced from hybridization between parental lines, F_1-F_4 generations over three years in field and controlled conditions of the National Phytotron Facility, IARI. Seeds from each selfed plant were harvested separately for advancing and progeny testing of the breeding population.

Pathotye of Puccinia triticina f.sp. *tritici*

The single spore culture of the race 77-5(121R63-1), one of the most virulent and predominant pathotypes of *P. triticina* in India was obtained from the Directorate of Wheat Research, Regional Station, Flowerdale, Shimla for phenotyping of different plant materials for their sensitivity to the leaf rust pathogen.

DNA extraction

Two to three healthy leaves were collected from each plant before inoculating it with leaf rust. The lyophilized leaf samples were ground to a fine powder with liquid nitrogen in a mortar and pestle and 50 mg of each powdered sample was used for DNA extraction using micro-extraction method as described by Prabhu *et al.* (1998) [8]. The DNA was diluted to a final concentration of 5 ng/µl for RAPD and 10 ng/µl for SCAR analysis.

Seedling test in the greenhouse

About 8-10 day old seedlings, DC 11[9], were inoculated during the evening hours. Prior to inoculation, the plants were sprayed with water to provide a uniform layer of moisture on the leaf surface. After inoculation, the seedlings were incubated for 36 h in humid glass chambers at a temperature of 23 ± 2°C and more than 85% relative humidity after which, the pots were shifted to muslin cloth chambers at the same temperature. The disease reaction was recorded 12-14 days after inoculation, using the scoring method described by Stakman *et al.* [10].

Adult plant testing in the greenhouse

The adult plants of breeding material (parents, F_1 and F_3 generations) were tested for infection types in the greenhouse and growth chamber at NPF, IARI, New Delhi. The growth stage for APR studies was fixed at stage 49 of Zadoks growth scale [11]. The middle part of two flag leaves of each plant was marked (an area of 2.5 cm^2 of each leaf) over which the inoculum was applied on the abaxial side of the leaf. The inoculated leaves were covered with moist plastic bag and incubated for 36 h at 25-18°C day-night regime. Reaction to leaf rust infection in greenhouse was recorded after 12 days of inoculation. The maximum infection type of two leaf samples per plant was always considered as the disease reaction by adopting the scoring method described by Stakman *et al.* [10].

Field testing

The F_2 and F_4 generations were field-tested using infector rows spaced as two rows after every twenty rows of 2.5m each of test material. Each block of breeding material was surrounded by infector row on all four sides planted ten days before the breeding material. Spores were sprayed as a suspension in water fortified with Tween20 (0.75 µl/ml) at an average concentration of 20 urediospores/microscopic field (10x x 10x).

PCR Amplification with SCAR marker

The specific primer pair of the SCAR marker $SCS1302_{607}$ described in Table 1 was used in the SCAR marker for amplification carried out in 25 µl reaction volume containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 100 µM of each dNTP (MBI Fermentas Germany), 0.75 unit Taq DNA polymerase (Bangalore

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Genei Pvt. Ltd., India), 10-20 ng of each primer and 20-40 ng of genomic DNA. Amplification reactions were performed in PTC-200 Thermal Cycler (MJ Research, USA) with the following thermal profile: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (primer annealing) and 72°C for 2 min (primer extension), with a final extension at 72°C for 7 min. PCR products were resolved on 2% agarose gel at 80 V for 3.5h.

PCR Amplification with RAPD markers

The random sequences of 10-mer oligonucleotide primers (Table 1) were produced from Biobasic Inc., Canada. The primers ($S3_{450}$ and $S336_{775}$) were selected on the basis of the RAPD linkage map developed in this laboratory for *Lr48* on 4BL by Maleki Zanjani and used for screening using the method described by Williams *et al*. (1990) [12] for RAPD. PCR amplification was performed in 20 µl reaction volume containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM MgCl2, 200 µM of each dNTP (MBI Fermentas, Germany), 0.75 unit Taq DNA Polymerase (Bangalore Genei Pvt Ltd, India), 0.2 µM of primer and 10-15 ng of genomic DNA. PCR amplification was achieved in PTC-200 thermal cycler (MJ research, USA) with the following thermal profile: one cycle of 2 min at 95°C (initial denaturation), followed by 45 cycles of 30 sec at 94°C (denaturation), 1 min at 36°C (primer annealing) and 1 min at 72°C with a ramp of l°C/ sec (primer extension), and 7 min at 72°C (a final extension). PCR products were size-fractionated on 2% agarose gel at 80V for 3.5h.

Statistical analysis

Segregation ratios were analyzed using chi-square test. The individuals of the crosses for the test of allelism of APR and seedling resistance that were scored as resistant and susceptible in the progeny populations were subjected to chi-square test for goodness of fit to test the deviation from the theoretically expected Mendelian segregation ratios. The molecular combination in progeny populations was also subjected to chi-square test for absence and presence of the marker fragment.

Results and discussion

It is acknowledged that leaf rust control could be most effective if APR is utilized in combination with seedling resistance in wheat breeding programs [13]. The most prominent example of the interactive complementary effects resulting in enhanced reaction of APR gene in the presence of seedling resistance genes is the case of durability of the resistance by the APR gene *Lr34* in the presence of seedling resistance [14].

MAS under field conditions in F_2 generation

The current experiment was an attempt to pyramid not only APR with seedling resistance, but also recessive with dominant genes which would not have been accomplished without employing MAS. Between the parental cultivars employed in these studies, the cultivar PBW343 based NIL performs agronomically well [15]. The disease reaction of the parent PBW 343 + Lr24 was resistant 0; 1- type at both seedling and adult plant stage while that of CSP44 was susceptible infection type 33+ at seedling and 0; at Zadoks stages 49 and above. The cultivar CSP44 (donor of *Lr48*) is not agronomically suitable in India [6]. Though little is known about the inheritance of APR, a few studies have reported that expression of APR was highly influenced by other *Lr* genes present in the background of the host genotype [16, 17]. Adult plant resistance expression to leaf rust has also been reported to be affected by environmental factors like light, temperature and growth stage [18, 19]. Since the seedling resistance gene *Lr24* in this case

Table 1. Details of the RAPD and SCAR markers selected as linked to the leaf rust resistance genes *Lr48* and *Lr24* in wheat

S.No.	Type of marker		Gene tagged	Sequence (5'-3')	Fragment/ product size	Linkage phase	Annealing temp °C
	SCAR	RAPD					
1		S3	*Lr48*	CAT CCC CCT G	450	Repulsion	36
2		S336	*Lr48*	TCC CCA TCA C	775	Coupling	36
3	SCS1302		*Lr24*	For: CGC AGG TTC CAA ATA CTT TTC Rev: CGC AGG TTC TAC CTA ATG CAA	607	Coupling	58

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remained consistent till adult stage and the APR gene *Lr48* functioned with a hypersensitivity reaction type in contrast to a lowered severity type as in other APR genes [6], there was no interference noticeable in the phenotypic expression of resistance in the selected plants with the pyramided genes over three generations. Therefore, the selection in the pyramided lines had to be based also on the phenotypic characters that have agronomic potential such as optimum tiller number, grain quality, yield and maturity period. In addition, since recessive APR was being pyramided with dominant seedling resistance to leaf rust, it was essential to standardize phenotyping along with molecular marker validation to provide consistency to the pyramiding program.

All the F_1 plants were resistant at seedling as well as 0; at adult plant stage. The SCAR marker $SCS1302_{607}$ linked to the gene *Lr24* in parent PBW 343 + Lr24 and the two RAPD markers - $S3_{450}$ (repulsion phase) and $S336_{775}$ (coupling phase) linked to *Lr48* locus in parent CSP44 were validated in the parents and control for their polymorphism to facilitate MAS (Fig. 1). All the F_1 plants amplified the polymorphic markers (Fig. 2). The two markers $S3_{450}$ and $S336_{775}$ linked as flanking markers were analyzed together as one unit against the SCAR marker $SCS1302_{607}$ for segregation on the 178 F_2 plants (Fig. 3). The molecular markers linked to the genes were crucial to achieve pyramiding and especially to identify the potential APR gene in the progeny plants whose presence is masked by the dominant seedling resistance gene. Selection efficiency for *Lr48* was improved by using coupling ($S3_{450}$) - and repulsion ($S336_{775}$) - flanking RAPD markers together as a codominant pair of markers in segregating populations where negligible double recombinants would occur [8].

Out of the 300 F_2 seeds sown for raising F_2 generation, 178 plants were left in the field due to unexpected rain resulting in water logging. From out of the 178 plants which were scored for disease at adult stage, 157 did not develop any leaf rust while 21 plants showed high level of susceptibility which recorded rust

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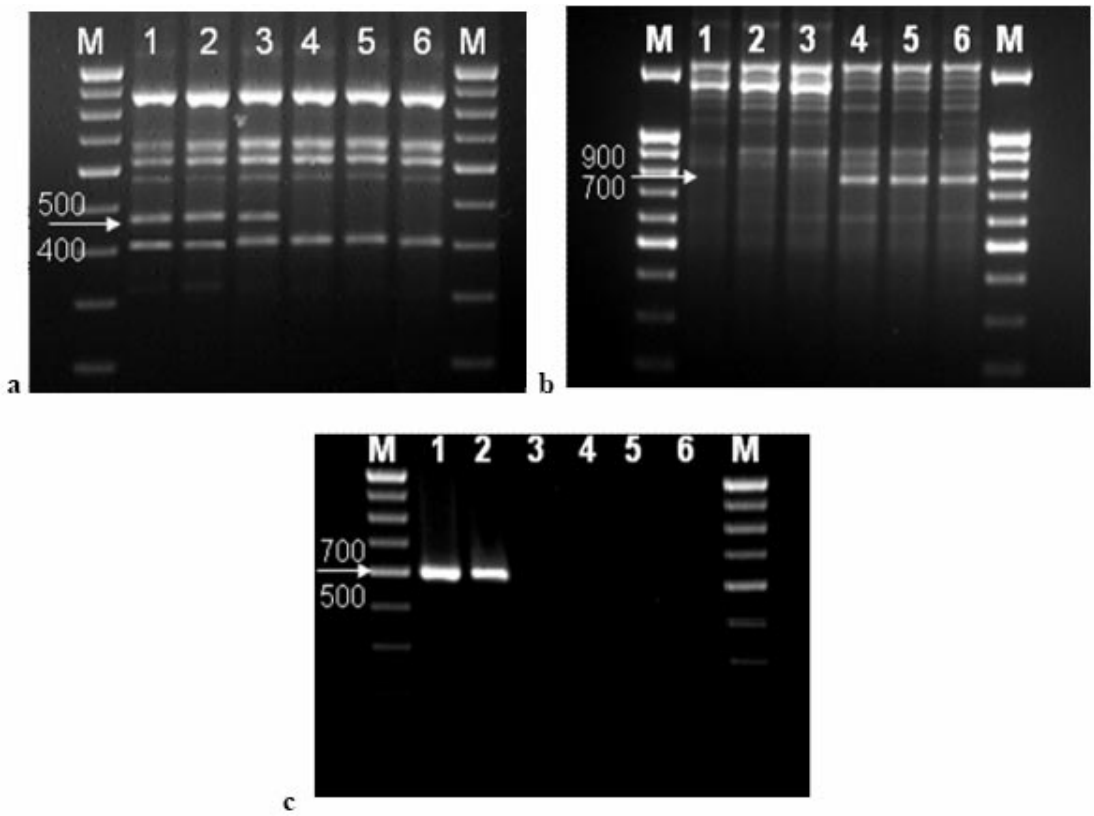

Fig. 1. Validation of (a) RAPD marker S3450 (repulsion phase) and (b) RAPD marker S336775 (coupling phase) linked to the gene *Lr48* in CSP44, (c) SCAR marker SCS1302607 linked to the gene *Lr24* in PBW343+Lr24 (M: Molecular weight marker, Lanes 1-2: a & b (CSP44), c (PBW343+*Lr24*), 3: a & b (CSP44), PBW343+*Lr28*), 4:a & b(PBW343+*Lr24*) and c(PBW343+*Lr28*), 5:a & b Parental line Thatcher+Lr25, 4: a, b & d (PBW343+*Lr24*) and c (PBW343+*Lr28*), 5: a & b (PBW343+*Lr28*), c(CSP44), 6: Agra Local (Susceptible variety)).

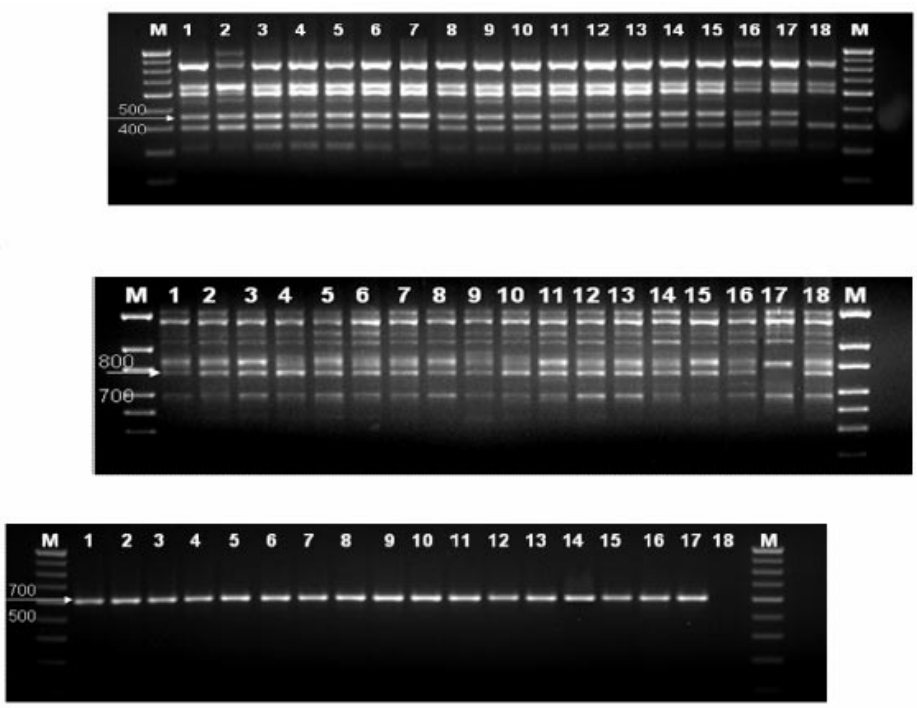

Fig. 2. Amplification of (a) RAPD marker $S3_{450}$ and (b) RAPD marker $S336_{775}$ linked to the gene *Lr48,* (c) SCAR marker $SCS1302_{607}$ linked to the gene *Lr24* in the F_1 plants of the cross PBW343+Lr24 X CSP44 (M: Molecular weight marker, Lanes 1-16: Presence of the linked marker fragment (arrow), Lane 17: a & b (CSP44) and c (PBW343+Lr24), 18: a & b (PBW343+Lr24) and c (CSP44)).

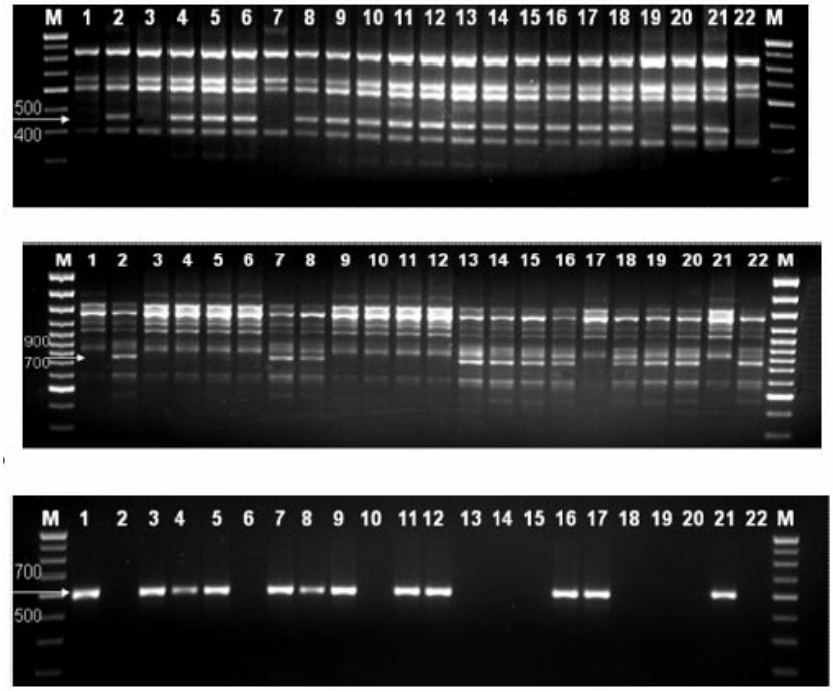

Fig. 3. F_2 population screening of leaf rust resistance / susceptible individuals with (a) RAPD Marker $S3_{450}$ and(b) RAPD marker $S336_{775}$ linked to the gene *Lr48,* (c) SCAR Marker $SCS1302_{607}$ linked to the gene *Lr24* in the cross (PBW343+Lr24) X (CSP44) (M: Molecular weight marker, Lanes 1-20: Presence / absence of the linked marker fragment (arrow), Lane21: a & b (CSP44) and c (PBW343+*Lr24*), 22: a & b (PBW343+Lr24) and c (CSP44)).

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severity ranging from 70S to 90S (data not presented). Due to disease escaped susceptible plants, which often occurs in the field screening, the population did not follow the expected segregation pattern of 13 Resistant: 3 Susceptible for one dominant gene (*Lr24*) and one recessive gene (*Lr48*), deviating marginally ($\chi^2 = 5.647$, p = 0.014).

The markers $S3_{450}$, $S336_{775}$ and $SCS1302_{607}$ segregated in the expected ratio 3: 1 (Table 2). Since the two markers linked to the APR were in one linkage group, mapped within 8.6 cM (Maleki Zanjani, 2004), distribution of the markers $SCS1302_{607}$ and $S3_{450}$ as well as $SCS1302_{607}$ and $S336_{775}$ segregated independently in the 9: 3: 3: 1 ratio for +/+, +/-, -/+ and -/-. The loci $S3_{450}$ and $S336_{775}$ were nested together to test for the F_2 distribution along with *Lr24* linked marker with a view to identifying the probable individuals which will be **+** for $S3_{450}$, **-** for $S336_{775}$ (as one linkage group for the selection of recessive APR allele *Lr48*) and + for $SCS1302_{607}$ (for selection of *Lr24*) in the F_2 population. The 9/16 genotypes which amplified the *Lr24* linked marker $SCS1302_{607}$ got split into 3/16 for $S3_{450}$ (+), $S336_{775}$ (-) and 6/16 with + for both $S3_{450}$ and $S336_{775}$ markers (Table 3). The same process was followed for the other two sets of 3/16 frequency groups, one which amplified $SCS1302_{607}$ and the other which amplified the RAPD markers for working out the number of plants carrying the other *Lr* gene respectively. A ratio of 3: 6: 3: 1: 2: 1 was tested for the expected segregation in the F_2 population (Table 3).

MAS enabled limiting the number of selected plants having the dominant genotype + and + for markers $SCS1302_{607}$ and $S3_{450}$ respectively as well as homozygous for marker $S336_{775}$ which resulted in the selection of 38 individuals. However, for selection, field-testing was effectively used for morphological assessment of the desirable plants which were identified as carrying the two genes based on molecular markers. While several plants that did not show the disease were detected to be disease-escapes by molecular markers, none of the susceptible plants was misclassified as resistant by the molecular markers validating the utility of the marker-assisted selection (Table 2). Among the resistant and marker positive plants tested for the three markers, those showing late maturity, lower grain number and tiller number than the parent (PBW 343 + *Lr*24) were rejected as desired in the breeding programme. The data generated was genetically equivalent to that obtained from a single co-dominant marker which enabled to select 38 F_3 families which were fixed for *Lr48* necessitating selection of progeny families homozygous for *Lr24* only in the F_3-F_4 generations (Table 3).

Table 2. Segregation pattern of F_2 generation in the cross PBW343 + *Lr*24 x CSP44 for markers SCS1302, S3 and S336 at each locus

Markers	+	-	Total	$\chi^2_{3:1}$	P
$SCS1302_{607}$	128	50	178	0.9064	0.3411
$S3_{450}$	144	34	178	3.3033	0.0691
$S336_{775}$	130	48	178	0.3671	0.5446

+ :Presence of band , - : Absence of band; $\chi^2_{9:3:3:1}$ for SCS1302 & S3 ($\chi^2 = 5.5081$ & P = 0.1382); $\chi^2_{9:3:3:1}$ for SCS1302 & S336 ($\chi^2 = 3.0658$ & P = 0.3816); $\chi^2_{9:3:3:1}$ for S3 & S336 ($\chi^2 = 17.6607$ & P= 0.0005)

Table 3. Genetic Analysis of joint segregation (*) of the three markers in the expected 3:6:3:1:2:1 ratio for the presence and absence of the marker fragment in F_2 population of the cross PBW343 + Lr24 X CSP44

SCS1302	S3	S336	Ratio (**)	Observed	Expected	χ^2	P
+	+	-	3/16	38	33.4	0.6335	0.4261
+	+	+	6/16	63	66.8	0.2167	0.6416
+	-	+	3/16	27	33.4	1.2263	0.2681
-	-	+	1/16	7	11.1	1.5144	0.2185
-	+	+	2/16	33	22.2	5.2541	0.0219
-	+	-	1/16	10	11.1	0.1090	0.7413
Total			16/16	178	178	8.9540	0.0299

*: Clustered segregation analysis between *Lr24* locus and *Lr48* locus inclusive of repulsion and coupling phase marker linked to *Lr48*.

**: Joint segregation ratio expected between SCS1302 and S3 pair and SCS1302 and S336. Double recombination among S336 and S3 flanking *Lr48* presumed to be absent

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MAS in F_3 generation greenhouse

In order to accommodate in the limited greenhouse space, only 15 out of 38 F_2 plant progeny accounting for the morphological desirability (F_3 families) were carried forward to generate F_3 family populations. Three of these families (104, 167, 248) were found to be homozygous for the seedling resistance at locus *Lr24* on the basis of the amplification of the gene specific SCAR marker $SCS1302_{607}$ (Fig. 4). This enabled rejection of 12 segregating progeny families within 3 weeks of seedling growth. The SCAR marker $SCS1302_{607}$ that specifically identified the gene *Lr24* complemented the pyramiding exercise by it being inseparably linked to the gene [7]. Liu *et al.* (2000) [20] employed 11 homozygous F_3 plants for powdery mildew resistance pyramiding by employing co-dominant SSR and RFLP markers.

Selection and field evaluation in F_4 generation

Thirty F_4 sub family plots representing 10 single plant progeny from each F_3 family were subjected to selection for the plant type accounting to traits like plant height, number of seeds per spike, number of tillers per plant and single plant yield in the field. Out of the three families, two families were found desirable for further pedigree selection considering the number of seeds available per plant. Twenty F_4 sub-families from two F_3 families (number 104 and 248) were exposed to selection based on a few traits of agronomic importance and screened with markers (Fig. 5). Further, a total of 26 single plants which amplified all the three marker fragments were tagged for generating F_5 generation to evaluate plant height, grain number, tiller number and yield along with resistance to leaf rust. This variation suggested scope for selecting for yield among the pyramided lines.

In the current study, the SCAR marker enabled the identification of 3 of the 15 F_3 families, which were already fixed for *Lr48*, as homozygous for *Lr24* also, thus successfully pyramiding the two genes. Once the genes conferring resistance to the same pathogen are tagged by tightly linked and reliable PCR based markers, they could relatively easily be accumulated into a single genotype via marker-facilitated selection [21]. This was best exploited in the current study. The segregation consistency in the F_2 and F_3 generations of the molecular markers confirmed the utility of MAS strategy where phenotyping is not stable, as was the case in the F_2

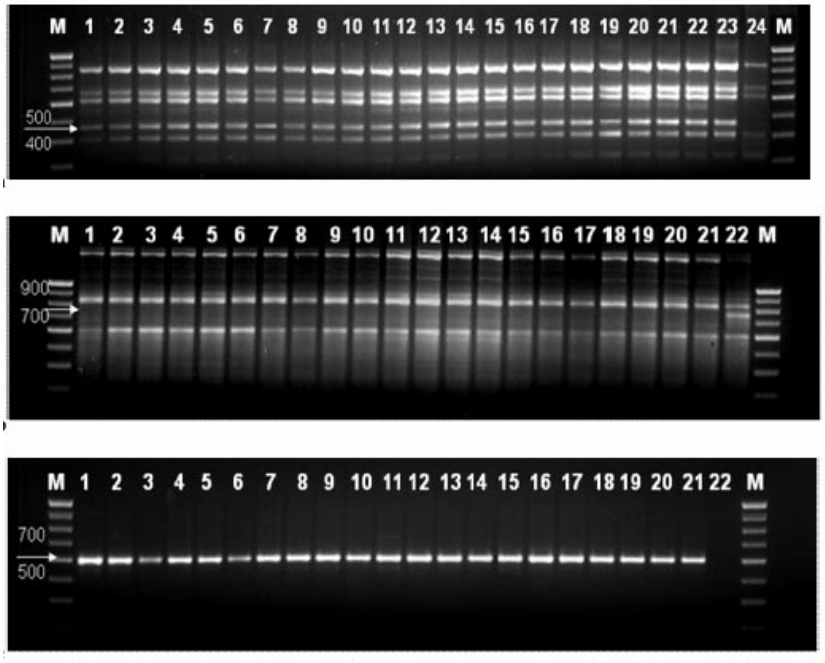

Fig. 4. An example of MAS identified homozygous F_3 family (#104) for (a) RAPD Marker $S3_{450}$, (b) RAPD Marker $S336_{775}$ linked to the gene *Lr48,* (c) SCAR Marker $SCS1302_{607}$ linked to the gene *Lr24* in the cross (PBW343+Lr24) x (CSP44) (M: Molecular weight marker, Lanes 1-22 in (a), 1-20 in (b) and (c) indicate presence (a & c) or absence (b) of the linked marker fragment (arrow), Lane21: b (CSP44), c (PBW343+Lr24), 22: b (PBW343+Lr24), c (CSP44), 23 : a(CSP44), 24: a (PBW343+Lr24)).

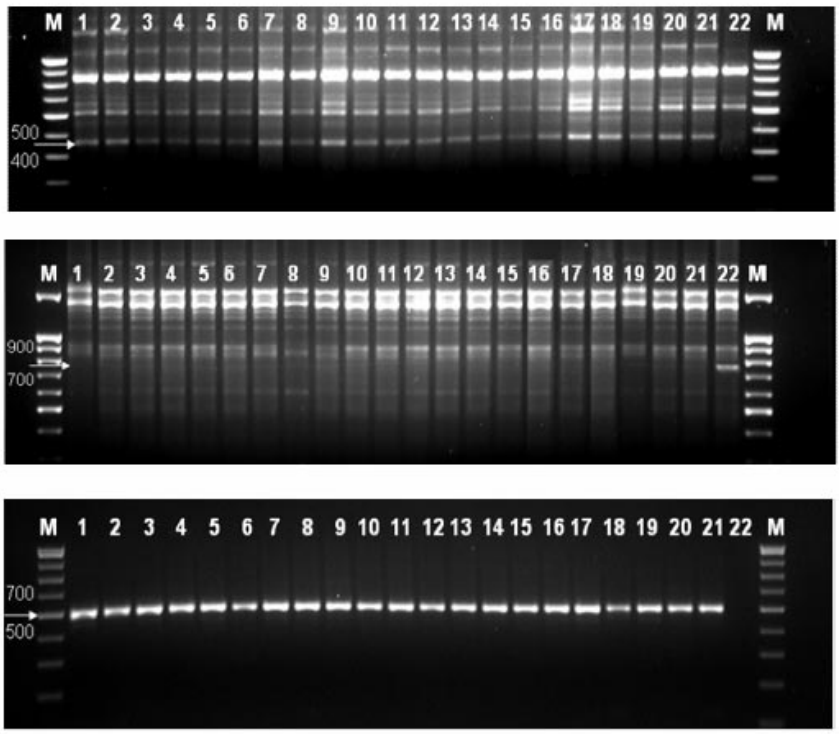

Fig. 5. Confirmation of MAS in the homozygous F_4 plants with (a) RAPD marker $S3_{450}$ and (b) RAPD Marker $S336_{775}$ linked to the gene *Lr48*, (c) SCAR Marker $SCS1302_{607}$ linked to the gene *Lr24* from the cross (PBW343+Lr24) x (CSP44) (M: Molecular weight marker, Lanes 1-20: presence (a & c) / absence (b) of the linked marker fragment (arrow), Lane 21: a & b (CSP44), d (PBW343+Lr24), 22: a & b (PBW343+ Lr24), d (CSP44)).

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generation where several F_2 rust susceptible plants escaped the disease despite congenial conditions which recorded up to 90S disease severity. The 26 leaf rust resistant pyramided lines in F_5 generation would be bulk tested for their agronomic suitability against the high yielding line PBW343 in the coming seasons before making final selection of the best few lines for yield trial and potential commercialization.

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