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Marker assisted gene pyramiding of leaf rust resistance genes *Lr* 9, *Lr* 24 and *Lr* 28 in a bread wheat cultivar HD 2329

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Abstract

Three highly effective alien genes for leaf rust resistance, *Lr 24*, *Lr 28* and *Lr 9* were selected for pyramiding in the background of a susceptible but well adapted bread wheat variety HD 2329. Screening against most virulent pathotypes of leaf rust 77-5 (121R63-1) indicated that all the three genes confer a high degree of seedling and adult plant resistance. The use of molecular markers, namely, $SCS1302_{607}$, $SCS421_{570}$ and $SCSS5_{550}$ validated the presence of the linked genes resistance genes, *Lr 24, Lr 28* and *Lr 9* in the three rust resistant near isogenic lines (NILs) of HD 2329. The application of molecular markers facilitated identification of individual plants in three-way cross (HD 2329+*Lr 24* x HD 2329+*Lr 28*) x HD 2329 + *Lr 9*, F_1 and F_2 generations possessing the targeted genes. Finally, one bulked progeny in F_8 generation was identified carrying the desired resistance genes, *Lr 24, Lr 28* and *Lr 9* in homozygous condition in the background of HD 2329. The availability of a combination of the three major rust resistance genes in desirable background would facilitate the strategic deployment of wheat varieties to achieve durable resistance.

Key words: *Triticum aestivum*, pyramiding, leaf rust, stripe rust, resistance genes, molecular markers

Introduction

Among several constraints towards realizing the potential yield in wheat, the rust diseases pose major threat to wheat production worldwide including India. All the three rusts of wheat, stem rust caused by *Puccinia graminis* Pers.f.sp. tritici Eriks.& Henn., leaf rust incited by *Puccinia triticina Eriks.* (Syn: *Puccinia recondita*) and stripe rust caused by *Puccinia striiformis* Westend., are occurring in designated wheat zones in India and cause significant losses in wheat production. Chemical control of rust pathogens is inefficient, expensive and cannot be adopted by small and marginal farmers. Hence the development of genetic resistance to rusts in host is advocated which is economical, effective and eco friendly to prevent the losses caused by rust epidemics (Kolmer, 1996). Although conventional gene transfer offer useful means of introgressing or pyramiding more than one well characterized resistance gene into susceptible genetic background. However, when no distinguishing virulence available for pyramiding two effective major genes, conventional technique is not useful for precisely pyramiding these genes in single genetic background. In such situations, gene transfers assisted by molecular techniques will be instrumental. Several rust resistance genes are available in the common wheat background originating from *Triticum* and its wild relatives like, *Agropyron, Aegilops* and *Secale.* A greatest hope for improving the rust resistance in wheat lies in exploiting some of these valuable species possessing useful genes. As many as 65 *Lr* genes have been reported in wheat and its relative's wheat (McIntosh *et al.*, 2008). 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 (Zadoks, 1961), seedling resistance is expressed on first leaf stage and remains effective throughout the growing period (Johnston and Melchers, 1929; Sambroski, 1986; Gospondinpva, 1987).

Many of the single genes, especially those which are effective throughout the crop growth stages of wheat, when incorporated singly become ineffective due to the constantly evolving new virulent forms of the leaf rust pathotypes, within few seasons of cultivation due to the heavy selection pressure imposed by the resistant varieties on the pathogen. Therefore, it is important to build a strategy that provides for a greater durability of the resistance in a variety. 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 (Roelfs, 1988). In wheat, to prevent the rapid breakdown of seedling resistance genes, it is suggested that such genes should be used in combinations as pyramids of resistant genes (Roelfs *et al.*, 1992; Jiang *et al.*, 1994). The gene *Lr* 9 derived from *Ae. umbellulata* is a highly effective gene throughout the world except in North America (Shaner *et al.*, 1972) and in Canada (Samborski and Dyck 1976)

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where pathogen races virulent on it have been reported. *Lr* 9 has been successfully transferred to different Indian bread wheat cultivars by repeated backcrossing using Abe as donor parent (Tomar and Menon, 2001). Another important gene *Lr* 24 is tightly linked to stem rust resistance gene *Sr* 24 on the long arm of the chromosome 3D (McIntosh *et al*., 1976). Both resistance genes (*Lr* 24 and *Sr* 24) were introduced from *Ag. elongatum* in a spontaneous translocation involving 3Ag from *Agropyron* and 3DL from wheat. Leaf rust resistance gene *Lr* 28 was transferred into bread wheat from *Ae. speltoides* while attempting to transfer *Yr8*, a gene for stripe rust resistance from *Ae. comosa* (Riley *et al*., 1968). In India, this gene has been used to create isogenic lines in different cultivars suited to different agro-climatic regions of the country and is effective against a wide spectrum of leaf rust races prevalent in India (Tomar and Menon, 1999). Pyramiding these effective resistance genes like *Lr* 9, *Lr* 24, *Lr* 28, is difficult to monitor in the field due to the inability to distinguish the expression of individual resistance genes and due to lack of availability of virulence in the pathogen to differentiate these genes. Marker-assisted pyramiding of these leaf rust resistance genes is being reported in the present investigation.

Material and methods

Plant material: The bread wheat genotype HD 2329, a variety with high agronomic adaptation covering more than 4 million hectares till early 1990s went out of cultivation due to its susceptibility to rust, was selected as recipient parent for pyramiding the leaf rust resistance genes, *Lr* 24, *Lr* 28 and *Lr* 9. Three near isogenic lines of HD 2329 namely HW 2037, which carries rust resistance gene *Lr* 24, HD 2329 + *Lr* 28, and HW 2055 which carries *Lr* 9 were utilized as NIL parents for a three way synthesis of HD 2329 + *Lr* 9 + *Lr* 24 + *Lr* 28. The F_1 hybrid between HD 2329 + *Lr* 28 and HW 2037 (NIL for *Lr* 24) was crossed with HW 2055 (NIL for *Lr* 9) and the three way F_1 plants were screened for the presence of resistance genes, *Lr* 24, *Lr* 28 and *Lr* 9 with the help of linked molecular markers. The desired crosses were made in controlled conditions of the National Phytotron Facility, IARI, New Delhi. Normal agronomical practices were followed for raising the crop. F_1-F_8 generations were grown over four years using 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.

Pathotype 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 reaction 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 micro-extraction method of DNA extraction as described by Prabhu *et al.* (1998). The DNA was diluted to a final concentration of 5ng/µl for RAPD and 10ng/µl for SCAR analysis.

Seedling test in the greenhouse: About 8-10 days old seedlings, DC 11(Zadoks *et al*., 1974), 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.* (1962). After recording the disease, infected leaves were removed so that the infection did not spread to the healthy leaves to facilitate fresh inoculation at adult stage.

Adult plant test 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 (Zadoks and Bouwman, 1985). 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 with the help of a camel hairbrush. The inoculated leaves were covered with moistured transparent plastic bag and incubated for 36 h at 25--18 ^{0}C day-night regime. After incubation the bags were removed from leaves. Reaction to leaf rust infection in greenhouse was recorded after 12 days of inoculation. The maximum infection type of the two leaf samples per plant was always considered as the disease reaction by adopting the scoring method described by Stakman *et al.* (1962). A second observation was recorded independently after two days of the first recording for eliminating bias and human error.

Field testing: The F_2 and F_4 generations were field-tested using infector rows planted as two rows after every twenty rows of 2.5m each of test material. Each block of breeding material was alsosurrounded 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×10x) as well as, inoculum's suspension was also injected into the last internode of the plant with the help of 2ml hypodermic syringe at boot leaf stage in the field before emergence of the boot (growth stage 49 of Zadoks *et al*., 1974). Field assessment of leaf rust severity was recorded based on the

modified Cobb's scale (Peterson *et al*., 1948) and recorded as percent severity of infection.

PCR amplification with SCAR marker: The SCAR marker $SCS421_{570}$, was used 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 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 thermal profile: initial denaturation at 94°C for 2 min, followed by 35 cycles at 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 per cent agarose gel at 80 V for 3 1/2 h.

Results and discussion

MAS under field conditions in F_2 generation: The recipient genotypes HD 2329, and the three NILs HD 2329 + *Lr* 24 (HW 2037), HD 2329 + *Lr* 9 (HW 2055) and HD 2329+*Lr*28, were screened with the help of SCAR markers linked to leaf rust resistance genes *Lr* 24, *Lr* 28 and *Lr* 9. These markers consistently amplified the specific marker fragments of 607bp linked to *Lr* 24 *in HW 2037*, 550bp linked to *Lr* 9 in HW 2055 and 570bp linked to *Lr* 28 in HD 2329 + *Lr*28 NIL confirming the presence of resistance genes in the donor parents (Fig 1 a, b and c). Testing with leaf rust pathotype 77-5 revealed a high level of seedling resistance in the three donor parents exhibiting infection type (IT) '0;' and the recurrent parent genotype HD 2329 showed susceptible reaction with the infection score of '3+' HD 2329 gave an 80S reaction to leaf rusts under field condition. A total of 205 three way F_1 plants were phenotyped for the presence of resistance genes, *Lr* 24, *Lr* 28 and *Lr* 9 and plants and the resistant plants were assessed with molecular markers. However, since the attempt was for three gene pyramiding and homozygosity achievement, a minimum of 50 seeds per plant was kept for advancing each selected plant for further breeding. A total of 98 leaf resistant plants with sufficient seeds were analysed with molecular markers linked to *Lr*24, *Lr*28 and *Lr*9 and 77 plants were identified to be carrying either two or three rust resistance genes (Table 1). The selected F_1 plants of the cross amplified the marker fragment $S5_{550}$ linked to *Lr* 9, $SCS421_{570}$, linked to gene *Lr* 28 and $SCS1302_{607}$ SCAR's linked to gene *Lr* 24 (Fig 1a,b,c). Out of these plants, 41 were identified linked to all the three genes *Lr*9, *Lr*24 and *Lr*28 by amplifying the critical marker fragments (Table 1). The other 36 plants possessed the two gene combinations. Hence, the SCAR marker $SCS5_{550}$ linked to *Lr* 9 (Gupta *et al*., 2005), $SCS1302_{607}$ linked to *Lr* 24 (Gupta *et al*., 2006) and $SCS421_{570}$ linked to *Lr* 28 (Cherukuri *et al*., 2005) were effective in identifying potential presence of the genes.

M: Molecular weight marker, Lanes 1-2: a (HW 2037+*Lr* 24, PBW343+*Lr*24), b (HD 2329+*Lr*28, PBW343+*Lr*28) and Lanes 1: c (HW 2055+*Lr* 9), Lane3: Parental line thatecher+*Lr25*, Lane 4: b & c (HW 2037+*Lr* 24) and a (HD 2329+*Lr* 28), Lane 5: a (HD 2329+*Lr* 28), b (HW 2037+*Lr* 24) & c (thatecher+*Lr* 9), and lane 6: Agra Local (Susceptible check).

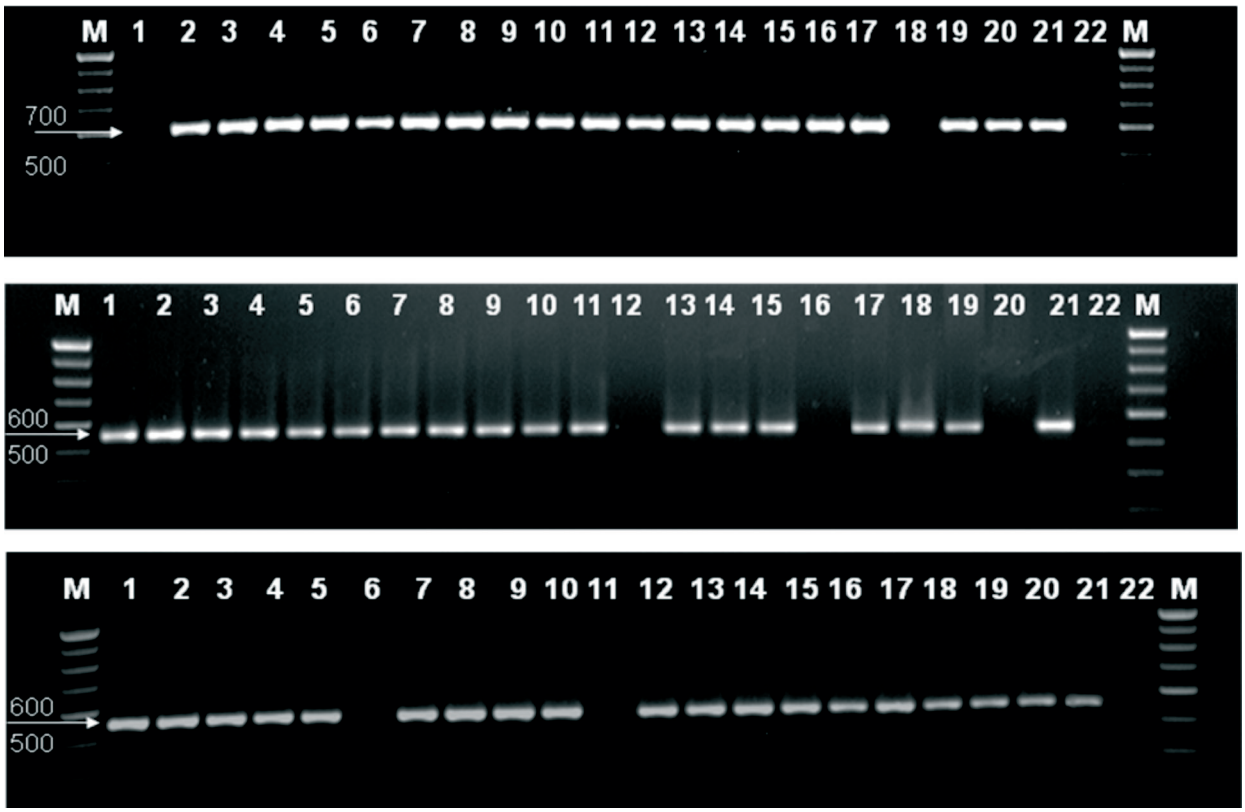
Fig. 1. Validation of (a) SCAR marker SCS1302607 linked to the gene *Lr* 24 in HW 2037and (b) SCAR marker SCS421570 linked to the gene *Lr* 28 in HD 2329+*Lr* 28, (c) SCAR marker SCS5550 linked to the gene *Lr* 9 in HW 2055.

Table 1. Marker assisted selection in segregating F_2 progeny of a cross developed for pyramiding *Lr* 9, *Lr* 24 and *Lr* 28 in HD 2329 background.

F_2 No.	S-5550 SCAR	SCS1302607 SCAR	S-421570 SCAR	*Lr* Genes carried	F_2 No.	S-5550 SCAR	SCS1302607 SCAR	S-421570 SCAR	Genes carried
1	-	+	+	24,28	52	-	-	-	None
2	+	-	-	9	53*	+	+	+	9,24,28
3 *	+	-	+	9,28	54*	+	+	+	9,24,28
4	-	+	+	24,28	55*	+	-	+	9,28
5 *	+	+	+	9,24,28	56	-	-	+	28
6	+	+	-	9,24	57	+	-	-	9
7 *	+	+	+	9,24,28	58	-	+	+	24,28
8	-	-	+	28	59*	+	-	+	9,28
9 *	+	+	+	9,24,28	60*	+	-	+	9,28
10*	+	+	+	9,24,28	61	-	+	-	24
11*	+	+	+	9,24,28	62*	+	+	+	9,24,28
12	-	+	+	24,28	63*	+	+	+	9,24,28
13*	+	+	+	9,24,28	64	+	+	-	9,24
14	-	+	+	24,28	65*	+	-	+	9,28
15*	+	+	+	9,24,28	66	+	-	-	9
16	+	-	-	9	67	+	-	-	9
17	-	-	-	None	68*	+	-	+	9,28
18	-	+	+	24,28	69	-	+	+	24,28
19*	+	+	+	9,24,28	70*	+	+	+	9,24,28
20	-	+	+	24,28	71*	+	+	+	9,24,28
21*	+	-	+	9,28	72	-	-	-	None
22*	+	+	+	9,24,28	73*	+	+	+	9,24,28
23*	+	+	+	9,24,28	74	-	+	+	24,28
24*	+	+	+	9,24,28	75*	+	+	+	9,24,28
25	-	+	-	24	76*	+	-	+	9,28
26*	+	+	+	9,24,28	77*	+	+	+	9,24,28
27*	+	+	+	9,24,28	78*	+	-	+	9,28
28	-	+	-	24	79*	+	+	+	9,24,28
29	-	+	+	24,28	80*	+	+	+	9,24,28
30*	+	-	+	9,28	81*	+	+	+	9,24,28
31*	+	-	+	9,28	82*	+	+	+	9,24,28
32*	+	+	+	9,24,28	83*	+	+	+	9,24,28
33	+	+	-	9,24	84*	+	+	+	9,24,28
34	-	-	+	28	85	-	+	-	24
35*	+	-	+	9,24,28	86	+	+	-	9,24
36*	+	-	+	9,28	87	-	+	-	24
37	-	-	-	None	88*	+	+	+	9,24,28
38*	+	-	+	9,28	89	+	+	-	9,24
39*	+	+	+	9,24,28	90*	+	+	+	9,24,28
40*	+	+	+	9,24,28	91*	+	+	+	9,24,28
41*	+	-	+	9,28	92	+	+	-	9,24
42	-	+	+	24,28	93	-	+	+	24,28
43	-	-	-	None	94*	+	+	+	9,24,28
44*	+	+	+	9,24,28	95	-	-	+	28
45*	+	+	+	9,24,28	96	-	+	-	24
46*	+	-	+	9,28	97	+	+	-	9,24
47	+	+	-	9,24	98	-	+	-	24
48*	+	+	+	9,24,28	HW 2055	+	-	-	9
49*	+	+	+	9,24,28	HW 2007	-	+	-	28
50*	+	+	+	9,24,28	HW 2037	-	+	-	24
51*	+	-	+	9,28	HD 2329	-	-	-	None

* Plants selected to have two gene (*Lr* 9+*Lr* 28) and three gene (*Lr* 9+*Lr* 24+*Lr* 28) combinations and carried forward for F3 generation, + Presence of respective marker band ; - Absence of respective marker band.

In the F_2 generation, DNA from each individual plant was screened for the markers and only those plants which amplified all the three critical marker fragments were selected for advancing (Fig 2 a,b,c). Thus, in total 41 F_2 plants were carried forward for generating the respective F_3 families and selecting potentially three gene-carrying lines. Sharp *et al.* (2001) and Babu *et al.* (2004) described that molecular markers linked to resistance genes were the best option for achieving selection for different gene combinations.



M: Molecular weight marker, Lanes 1-20:F_2 plants, lane 21: a & b and c (HW 2037, HD 2329+*Lr* 28, HW 2055), lane 22: a, b and c (HD 2329+*Lr* 28, HW 2037+*Lr* 24, HD 2329+*Lr* 28), respectively.

Fig. 2. Screening of F_2 population derived from three way cross with (a) SCAR marker $SCS1302_{607}$ linked to the gene *Lr* 24 in HW 2037 and (b) SCAR marker $SCS421_{570}$ linked to the gene *Lr* 28 in HD 2329+*Lr* 28, (c) SCAR marker $SCS5_{550}$ linked to the gene *Lr* 9 in HW 2055.

MAS in F_3 generation: In the F_3 generation all the 41 families were represented by 337 plants on which DNA analyses with respect to the three markers could be carried out. There was segregation observed for all three markers in eight of the 41 families, which were out rightly omitted from further advancement. One particular family showed no segregation for all the three markers $SCS5_{550}$, $SCS73_{719}$ and $SCS421_{570}$ indicating that this family might be homozygous for all the three genes. Single plants were identified from selected F3 families which showed amplification for all the three markers and only these plants were advanced to F_4 stage. Such plants totalled to 126 selected from 33 F_3 families. Among these, 21 single plant progenies were carried forward after analyzing the 126 plants with the SSR marker $Xgwm160_{180}$ that was genome specific marker of the *Ae. speltoides* fragment carrying the gene *Lr* 28 in the translocation source line. This dominant marker specifically identified homozygous plants at the *Lr* 28 locus. On the basis of the space and time allocation and availability of seed, it was decided to raise a minimum of 71 F_4 sub families for further advancement.

Successful marker-assisted pyramiding of disease resistance genes has already been reported in wheat with respect to three leaf rust genes *Lr13, Lr34* and *Lr37* (Kloppers *et al*., 1997) and three powdery mildew genes *Pm3, Pm4a* and *Pm21* (Liu *et al*., 2000). Sivasamy *et al* .(2009) successfully achieved multi gene combination in BC3-F5 generation by conventional breeding utilizing the advantage of linkage and confirmed the presence of genes involved in their study. They had used the linked genes (*Lr19/Sr 25* and *Sr 36/Pm6*), which facilitated easy transfer of targeted genes in different genetic backgrounds.

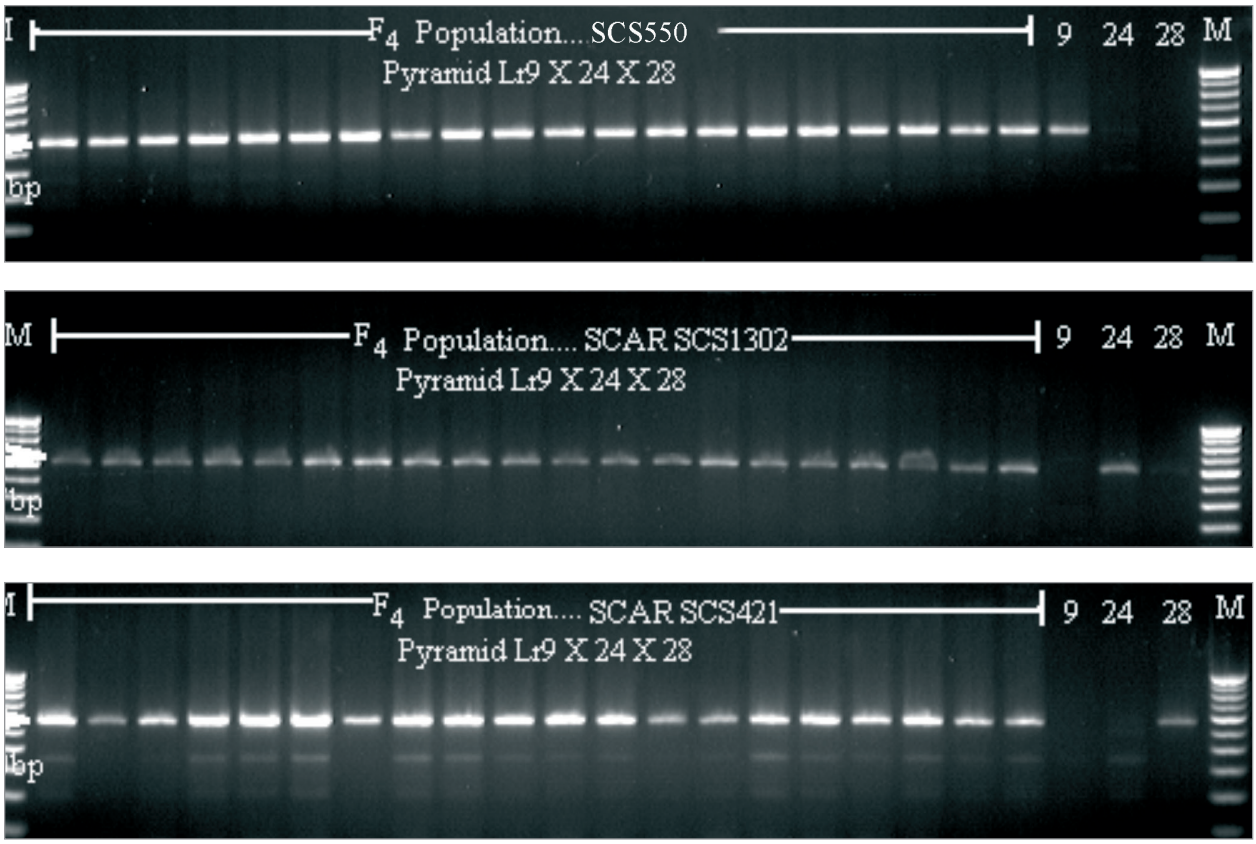


Fig. 3. Confirmation of homozygous F_4 plants with (a) SCAR marker $SCS5_{550}$ linked to the gene *Lr* 9 in HW 2055 and (b) SCAR marker $SCS1302_{607}$ linked to the gene *Lr* 24 in HW 2037, (c) SCAR marker $SCS421_{570}$ linked to the gene *Lr* 28 in HD 2329 (NIL for *Lr* 28) in the 3-way population (HD 2329, *Lr* 9, *Lr* 24, *Lr* 28).

Attempts have been made to pyramid resistance genes in rice with the objective of developing more robust genetic resistance (Huang *et al*., 1997; Sharma *et al*., 2004). In such an effort carried out earlier in our laboratory to breed for durable resistance, the seedling resistant gene *Lr* 24 has been pyramided along the APR gene *Lr48* through marker assisted selection approach (Samsampour *et al*., 2009).

Selection in advanced generations and field evaluation of pyramided lines: Single plants were evaluated for all the three genes from each selected F_3 family in F_4 generation. Progenies which were non-segregating for the marker linked to *Lr* 9, *Lr* 24 and *Lr* 28 (Fig. 3a, b and c) were selected.

A total of 25 single plants from each of 26 homozygous families were tagged for generating F_5 breeding population for selection on the basis of plant height, grain number, tiller number and yield components. From five pedigree selected single plants in F_7 generation, two lines have been selected for evaluation as essentially derived varieties (EDVs) of HD 2329 for registration under PPVFRA (Figure 4).

Since *Lr* 24 is linked with stem rust resistance gene *Sr* 24, the newly pyramided lines are expected to show additional resistance to stem rust as well. The *Lr* 28 gene remained effective for many decades in India and elsewhere, however, Bhardwaj *et al*., (2010) recently reported a new virulent pathotype 121 R60-1 from India, which appears closely related to the most prevalent pathotype 121R 63-1 (77-5). Nevertheless, the lines obtained with different combination of rust resistance genes in HD 2329 background are likely to provide enhanced durable resistance. These lines can be used for gene deployment after the testing of their yield potential at multi-locations. The material can also be used for molecular studies.

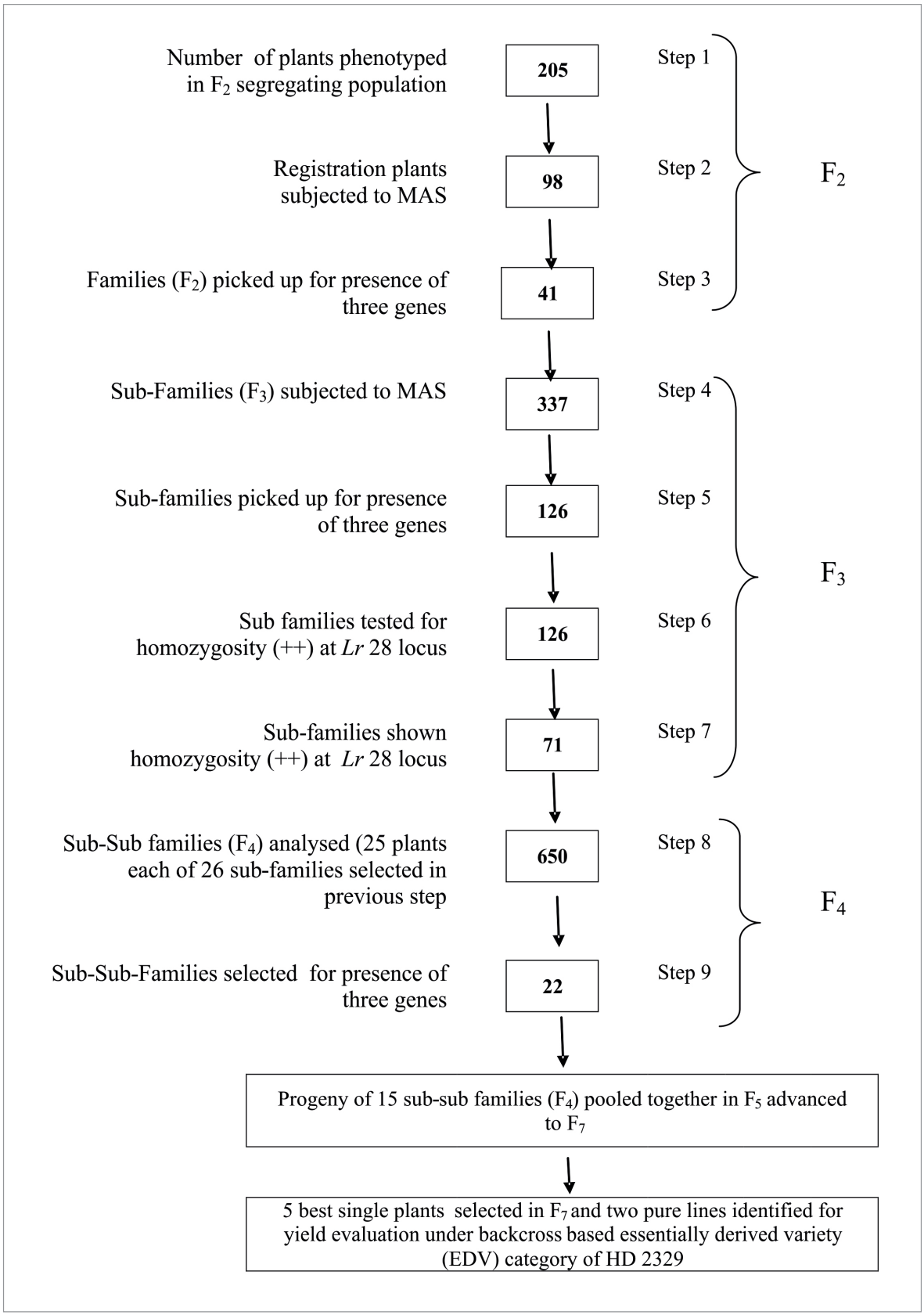


Fig. 4. Schematic representation of the pyramiding of three genes *Lr* 9, *Lr* 24 and *Lr* 28 in HD 2329 background using marker assisted selection.

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