RESEARCH ARTICLE

Marker-assisted pyramiding of *Thinopyrum*derived leaf rust resistance genes *Lr19* and *Lr24*in bread wheat variety HD2733

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Abstract

The study was undertaken to pyramid two effective leaf rust resistance genes (*Lr19* and *Lr24)* derived from *Thinopyrum*(syn.*Agropyron),* in the susceptible but agronomically superior wheat cultivar HD2733 using marker assisted selection. HD2733, released for irrigated, timely sown conditions ofthe North Eastern Plains Zone (NEPZ) of India in 2001, became susceptible to leaf rust,a major disease of the region. Background selection helped in developing near-isogenic lines (NILs)of HD2733 with *Lr19* and *Lr24* with 97.27and 98.94 percent genomic similarity withthe parent cultivar,respectively, after two backcrossing and one generation of selfing. NILs were inter-crossed to combine the genes *Lr19* and *Lr24*. The

combination of these two genes in the cultivar HD 2733 is expected to provide durable leaf rust resistance in farmers' fields.

Introduction

Rust diseases caused by *Puccinia*spp.inflict significant yield lossesto wheat crop throughout the world (Kolmer 2005; Tomar*et al*. 2014). There are three rusts, leaf rust (*Pucciniatriticina*), stem rust (*Pucciniagraministritici*) and stripe rust (*Pucciniastriiformis*). All three prevail in India but require different agro-ecological conditions for disease development. Leaf rust exists in all the wheat growing regions while stem rust is common in warmer areas of central and peninsular India. Stripe rust is a disease of cooler areas of North-western India including northern Himalayas. Development and deployment of rust resistant cultivars is the most economical, effective and environment friendly approach to prevent the damage caused by rust diseases (Kolmer 1996). So far, 76leaf rust resistance (*Lr*) genes have been designated (McIntosh *et al*. 2016). About half of them are native to the bread wheat *Triticumaestivum*but most are not effective to existing Indianraces.Therefore, alien genes for leaf rust resistance have been deployed in many cultivars which provide effective resistance. However, some of the alien leaf rust resistance genes such as *Lr9*,*Lr19*, *Lr26* and *Lr28*became ineffective due to evolution of new virulent races(Nayar*et al.* 2003; Bhardwaj *et al.* 2005, 2010). Of these, only*Lr26*hasbeen used extensively in wheat cultivars. In general, cultivars carrying single race specific resistance gene have been found to be short lived due to evolution of new races of pathogen. Therefore, pyramiding of rust resistance genes is considered an effective strategy for enhancing durability of resistance genes.

Wheat variety HD2733 is a high yielding variety released for cultivation under timely sown irrigated conditions of NEPZ in India.However, over period of time, this cultivar became susceptible to leaf rust. To enhance durability of HD2733, efforts were initiated to transfer rust resistance genes using marker assisted backcross breeding (MABB). While

conventional backcross method requires 6-7 generations to transfer targeted genes in a variety, MABB can achieve similar results in 2-3 generations of backcrossing (Pedersen andLeath 1988;Mallick*et al.* 2015). Moreover, identifying plants carrying the targeted resistance gene(s) in each backcross generation is easier using molecular markers than the conventional method of selection. In this paper, we report the accelerated transfer of rust resistance genes *Lr19* and *Lr24* using marker assisted foreground and background selection in wheat variety HD2733.Since *Lr19* and *Lr24* are linked with stem rust resistance genes *Sr25* and *Sr24* respectively, the pyramided lines are expected to carry themas well.

Materials and Methods

Plant materials

Wheat variety HD2733 was used as recipient parent. Donor genotypes included two backcross derived lines of breadwheat variety HD2687 carryingresistance genes for leaf rust,*Lr19* (HD2687+*Lr19*) and *Lr24* (HD2687+*Lr24*), respectively (Sivasamy *et al.* 2007).

Molecular markers and marker analysis

SSR marker *Xwmc221*andSCAR marker *SCS1302* wereused for marker assisted foreground selection of *Lr19* and *Lr24*, respectively(Gupta *et al*.2006 a,b). For background selection, 907 SSR markersspanning across 21 chromosomes of bread wheat were usedfor parental polymorphism. Recurrent parent genome (RPG) was calculated as described earlier (Mallick*et al.* 2015).

The polymerase chain reaction (PCR)wascarried outin 10µl reaction volumes with 25 ng of genomic DNA, 1.0 unit *Taq* DNA polymerase (Bangalore Genei Pvt Ltd, India),200µM of each dNTP (MBI Fermentas, Germany),0.2µM of both forward and reverse primers,4 mMTris- HCl (pH 8.0), 20 mMKCl, and 0.8mM $MgCl_2$. The PCR was performed in thermal

cycler (Eppendorf, model Mastercycler pro S, Hamburg, Germany) at temperature profile of 94ºC for 4 min, followedby 35 cycles of reaction having 94ºC for 30 sec,50–60ºC for 30 sec (annealingtemperature depending on primer) and 72ºC for 30 sec, with a final extension at 72ºC for 10 min.The amplified products were resolved on MetaPhor^{TM} (Lonza) agarose gel (3.5 %) and visualized by ethidium bromide staining.

DNAextraction

Leaftissuesfor genomic DNA isolationwerecollected after30-40daysof sowing. DNA was isolated by CTAB method(Murray and Thompson 1980) and quantified on0.8% agarose gel by comparing samples with 100ng/200ng of Lambda uncut DNA. Working DNA stockswereprepared by dilution in TE buffer to achieve final concentration of about25ng/µl forPCR amplification.

Marker assisted genepyramidingscheme

HD2733 was crossed with donor lines HD2687+ *Lr19* and HD2687+*Lr24* to produce two separate F_1 generations in 2009-10. F_1 plants were crossed with recurrent parent HD2733 to developBC_1F_1 generation. Marker assisted selection for *Lr19* and *Lr24*was carried outin respective BC_1F_1 generations.Gene positive plants in BC_1F_1 were subjected to background analysis. Plants with targeted leaf rust resistance gene with maximum RPG were backcrossed with recurrent parent HD2733 to generate two separate BC_2F_1populations. In BC_2F_1, foreground as well asbackground selection was practiced as done inBC_1F_1. BC_2F_1 plants with rust resistance genes and maximum RPG were selfedas well asinter-crossed to obtain $BC_2F_2$and $NILF_1$generations, respectively (Fig. 1). Marker assisted foreground selection identified plants homozygous for*Lr19* and those carrying *Lr24* in homozygous/heterozygous state, in respective BC_2F_2 populations. Selected BC_2F_2 plants with *Lr24* were selfed to

developBC$_2$F$_3$families. Non-segregatingBC$_2$F$_3$ families,carrying*Lr24* in homozygous state were identified by *Lr24*linked marker.Plants carrying single leaf rust resistance gene in homozygous state in BC$_2$F$_2$ generation were subjected to background analysisand plants with maximum RPG (NILs) were identified. The inter-crossed NILF$_1$ plants carryingboth *Lr19*and *Lr24*were selfed to produce NILF$_2$ generation. NILF$_2$population was raised and plants carrying *Lr19* in homozygous statealong with *Lr24*were identified with respective markers. NILF$_2$ plants homozygous for *Lr24* were identified on the basis of progeny testingin NILF$_3$.Thus, NILF$_3$families carrying both *Lr19* and *Lr24* in homozygous state were developed. The details of gene pyramiding schemeare givenin Fig 1.

Apart from the use of molecular markers, shuttle breeding was used to accelerate thedevelopment of NILs. Two generations in ayear were raised; one at IARI, New Delhi to grow main season crop in winter and the other at IARI, Regional Station, Wellington, Tamil Nadu to grow off-season crop in summer. The main season at Delhi was used to raise F$_1$, BC$_2$F$_1$, BC$_2$F$_3$, NILF$_2$, and NILF$_3$ generations while BC$_1$F$_1$, BC$_2$F$_2$ andinter-crossed NILF$_1$were grown in off season at Wellington.

Rustinoculation

Segregating material was tested with the most dominant leaf rust race 77-5 under artificial epiphytotic conditions. NILF$_3$s were tested in two isolated nurseries inoculated with mixture of leaf and stem rust races. The generations raised at Wellington were naturally exposed to leaf and stem rusts as Wellington (Nilgiri hills)is a natural hotspot for the two rusts (Nagarajan and Joshi 1980). The rust reactions were recorded at adult plant stage following modified Cobb Scale (Peterson 1948) where rust severity is recorded on 0-100 scale along with infection response as S = Susceptible, MS= Moderately Susceptible, MR= Moderately Resistant and TR= Trace (Joshi *et al.*1988).

Results

Genetic analysis of recurrent (HD2733) and donor parents (HD2687+*Lr19* and HD2687+*Lr24*)using 907 S*SR* markers identified 110 and 95 markers, respectively as polymorphic. Polymorphic markers were used for background selection of plants carrying targeted resistance genes. The number of plants subjected to background selection were minimised based on phenotypic similaritywith the recurrent parent HD2733.

In BC_1F_1(HD2733 /HD2687+*Lr19*//HD273), 62 plants were identified carrying *Lr19* in heterozygous state by marker *Xwmc221*. In the other BC_1F_1(HD2733 /HD2687+*Lr*24//HD2733),SCAR marker *SCS1302* identified 64 plants with*Lr24*(Table 1). All the selected plants were resistant in the field with no visible symptoms of leaf rust while the recurrent parent HD2733showed susceptiblereaction of 40S to 50S.Background selection with 110 polymorphicmarkers in *Lr19*positive plants in BC_1F_1recovered a maximumof81.03 percent recurrent parent genome (RPG). Similarly, background analysis with 95 polymorphic markers in the other BC_1F_1 population (HD2733/HD2687+*Lr*24//HD2733) identified maximum genomic similarity of 87.67 percentwith HD2733(Table 1). Two plants from each BC_1F_1 population with targeted gene and high RPG were backcrossed with HD2733 to minimize the risk of losing any relevant plant. In BC_2F_1, 30 plants carrying *Lr19* and 15 carrying *Lr24*, were identified (Table1). As expected, all the plants carrying*Lr19*were heterozygous in BC_1F_1 and BC_2F_1.However,for*Lr24*, the presence of the gene could be established in BC_1F_1 and BC_2F_1 generations since unlike *Lr19* marker *Xwmc221*, the SCAR marker for *Lr24* is a dominant marker.

In the BC_2F_1, recovery of HD2733 genome increased to 91.95 and 91.09 percentfor the *Lr19* and *Lr24* gene positive plants, respectively. In the BC_2F_2, foreground analysis revealed that 16plants out of 51 were homozygous for *Lr19* and 31 out of 55

carried*Lr24*,either in homozygous or heterozygous state. Background analysis showed maximum RPG of 97.27 percent for *Lr19* and 98.94 percentfor *Lr24* in BC_2F_2. In this way, two Near Isogenic Lines (NILs) of HD2733 carrying *Lr19* and *Lr24* individually, were developed. Screening of 40 $NILF_1$plants produced by inter-crossing the two $BC_2F_{1s}$for*Lr19* and *Lr24*with respective markers identified 7 plants carrying both the genes.These 7 plants were selfed to produce$NILF_2$ generation. Foreground analysis of selected 130 $NILF_2$ plants identified 53 plants carrying *Lr19* in homozygous condition along with *Lr24*.The two gene combination plants in $NILF_2$were subjected to background analysis which revealed a maximum RPG recovery of 98.57 percent. Of the 53 $NILF_2$ plants, 12 were found homozygous for both *Lr19* and *Lr24* based on progeny testing in $NILF_3$. The NILs carrying *Lr*19, *Lr24* and *Lr19+Lr24* were evaluated for leaf and stem rust resistance. All the NILs showed high degree of resistance to leaf rust with maximum disease reaction of TR to 10 MR. Additionally, NILs also showed resistance to stem rust, reaction ranging from 5S to 5 MR. The recurrent parent HD2733 showed susceptible reaction (40S) to leaf rust and moderately susceptible reaction (10MS) to stem rust (Table 2).

Discussion

HD2733 is a well-adapted and high yielding wheat variety inNEPZ of India. Since it became susceptible to leaf rust, its resistance was restored by pyramiding two resistance genes *Lr19* and *Lr24*. The *Thinopyrum* derived resistance gene *Lr19* (Sharma and Knott 1966) is linked with stem rust resistance gene *Sr25*. Singh *et al*. (1998) reported that 7D/7Ag translocation carrying *Lr19/Sr25*enhanced grain yield by 10-15 percentin several genotypes. The other *Thinopyrum* derived leaf rust resistancegene *Lr24* present on the long arm of 3D chromosome is linked with stem rust resistance gene *Sr24*(McIntosh *et al.* 1976). Thus, besides pyramiding of leaf rust resistance genes *Lr19* and *Lr24*, stem rust resistance genes *Sr25* and *Sr24*werealso

pyramided simultaneously. Additionally, stem rust resistance gene *Sr25* confersresistance to stem rust race Ug99 (Sharma *et al*. 2013).Though, race Ug99 is not present in India,it is desirable to incorporate genetic resistance against Ug99 to ward off future threats. The combination of *Lr19/Sr25 + Lr24/Sr24*confersresistance to all the prevalent races of leaf and stem rust in India. The first wheat variety carrying *Lr24* was released in 1993 and subsequently a large number of varieties with *Lr24* have been released in India. Though, virulence has been reported from several countries,*Lr24* continues to provide effective resistance in India (Tomar *et al.* 2014). For *Lr19,* arace (77-8)was reported as virulent. However, it is a weak race and despite of being reported in 2005, it has not spread in India. HD2733 pyramided lines carrying both *Lr19* and *Lr24*are expected to cross protect each other,thus enhancing their durability. *Lr24* does not seem to impose yield penalty as demonstrated by the fact that several cultivars with *Lr24* have been released for cultivation in India (Tomar*et al.* 2014).

Transfer of rust resistance genes by conventional backcross method requires screeningwith discriminating raceseither at seedling or adult stage under epiphytotic conditions. However, with the availability of robust molecular markers linked with resistance genes, it is possible to identify targeted resistance gene(s) in segregating populations with greater precision (Sivasamy*et al.* 2009;Revathi*et al.* 2010; Bhawar*et al*.2011). At the same time, background selection with molecular markers enables faster recovery of genome of the recipient variety.

In the present experiment, background selection using polymorphic markers was started right from BC_1F_1 generation. Phenotypic selection of plants similar to recurrent parent HD2733 followed by marker assisted background selection helped in faster recovery of RPG. Parental polymorphism between HD2733 and the two donor lines, HD2687+*Lr19* and HD2687+*Lr24* identified markers which helped in recovering alleles specific to HD2733 in

each backcross generation. The number of markers requiredfor background selection in BC_2F_1andBC_2F_2reduced considerablyas after each backcrossing some alleles of HD2733 gets fixed so that no further selection is required. Using MABB, it was possible to select desirableplants with high RPG along with resistance genes in each generation which in turn accelerated the development of near- isogenic lines of HD2733.

Marker assisted selection

Foreground selection was carried in BC_1F_1, BC_2F_1, BC_2F_2, $NILF_1$ and $NILF_2$ generations to identify plants with*Lr19* and *Lr24*.Plants homozygous for *Lr19* were identified in BC_2F_2 using co-dominant marker *Xwmc221*. However, SCAR marker *SCS1302* being dominant,it was not possible to identify plants homozygous for *Lr24*.Plants homozygous for *Lr24* were identified on the basis of progeny testing in BC_2F_3 generation. Near-isogenic lines carrying *Lr19* and *Lr24* individually were inter-crossed to combine targeted genes *Lr19* and *Lr24* in $NILF_1$. Marker assisted selection with *Lr19*and *Lr24*was also conducted in inter-crossed ($NILF_1$) plants as the combination cross was made in BC_2F_1 itself where both the genes were in heterozygous state. The plants identified in $NILF_1$ generation were heterozygous for both*Lr*19 and *Lr24*. In $NILF_2$, plants homozygous for *Lr19* and additionally carrying *Lr24* were identified using respective molecular markers. In $NILF_3$, families homozygous for *Lr24* were identified. Since these families were already homozygous for *Lr19*, it was possible to select the F_3 families carrying both *Lr19* and *Lr24* in homozygous state.

Markers assisted background selection accelerated the recovery of RPG. In BC_1F_1 derived from the cross HD2733/HD2687+*Lr*19//HD2733, maximum 81.03 percent genomic similarity with recurrent parent HD2733 was recovered. In the BC_2F_1, the genomic similarity increased to 91.95 percent which further increased to 97.27 percent in BC_2F_2. Thus, NILs carrying *Lr19* were producedwith two backcrosses followed by one generation of selfing. The

other leaf rust resistance gene *Lr24* was also transferred efficiently in the background of HD2733. Plants with highest RPG were identified in the BC_1F_1, BC_2F_1 and BC_2F_2 generations (Table 1). Thus, near isogenic lines of HD2733 carrying genes *Lr19* and*Lr24*with RPG of NILs reaching to 97.27 and 98.94 percent, respectively were developed. Phenotypic selection was usedin addition to marker assisted background selection which helped in recovering better plant types.

The NILs carryingindividualgenes *Lr19* and *Lr24*can be used in future for producing other gene combinations.Higher recovery of the HD2733 genome (more than 90 percentRPG) in the BC_2F_1 generation also enabled inter-crossing *Lr19*and *Lr24* carrying plants to produce two gene combinations. Combination of marker assisted foreground and background selection significantly reduced the period required to produce NILs carrying *Lr19* and *Lr24* individually. Two different approaches are recommended to pyramid two or more rust resistance genes in a popular cultivar. In the first approach genes from different donors are first combined in a single plant, followed by backcrossing with recurrent parent and selection of targeted genes. In the second approach which was followedin the present study, near-isogenic lines carrying individual genes are produced first, followed by inter-crossing of NILs to combine the targeted genes. Ishii *et al.* (2008) showed that the second approach where NILs are produced is superior to the first approach. The recurrent parent HD2733 carries 1RS.1BL translocation which carries *Lr26* and *Sr31*. *Lr26* is not effective in India, therefore, HD2733 showed susceptible reaction to leaf rust. However, moderately susceptible response (10MS) of HD2733 against stem rust (Table2) is due to the presence of *Sr31*.The NILs developed in the present study provide improved versions of HD2733 with high degree of leaf and stem rust resistance.

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Table 1. Number of gene positive plants and maximum RPG percent identified in different generations of HD2733

S.No.	Cross	Generation	Total no. of plants screened	No. of gene positive plants		No. of gene negative plants	Maximum RPG (%)
				Homozygous	Heterozygous		
1	HD2733/ HD2687+ *Lr19*	BC_1F_1	128	-	62	66	81.03
		BC_2F_1	75	-	30	45	91.95
		BC_2F_2	51	16	21	14	97.27
2	HD2733/ HD2687+ *Lr24*	BC_1F_1	134	-	64	70	87.67
		BC_2F_1	32	-	15	17	91.09
		BC_2F_2	55	31		24	98.94
		BC_2F_3	31	12	19	-	-
		Generation	**Total no. of plants screened**	**Number of plants with two gene combinations**		**No. of gene negative plants**	**Maximum RPG (%)**
				Homozygous	**Heterozygous**		
3	HD2733+ *Lr19*/HD2733+ *Lr24*	$NILF_1$	40	-	7	33	-
		$NILF_2$	130	53		-	98.57
		$NILF_3$	53	12	41		

Maximum rust infection		
Introgressed lines	**Leaf rust**	**Stem rust**
HD2733	40S	10MS
HD2733+*Lr19*/*Sr25*	10MR	15MS
HD2733+*Lr24*/*Sr24*	10MR	5S
HD2733+*Lr19*/*Sr25*+ *Lr24*/*Sr24*	TR	5MR

Table 2. Maximum rust reaction recorded in the recurrent parent HD2733 and selected near-isogenic lines

Figure 1. Marker assisted gene pyramiding scheme to combine leaf rust resistance genes*Lr19* and *Lr24* in wheat variety HD2733

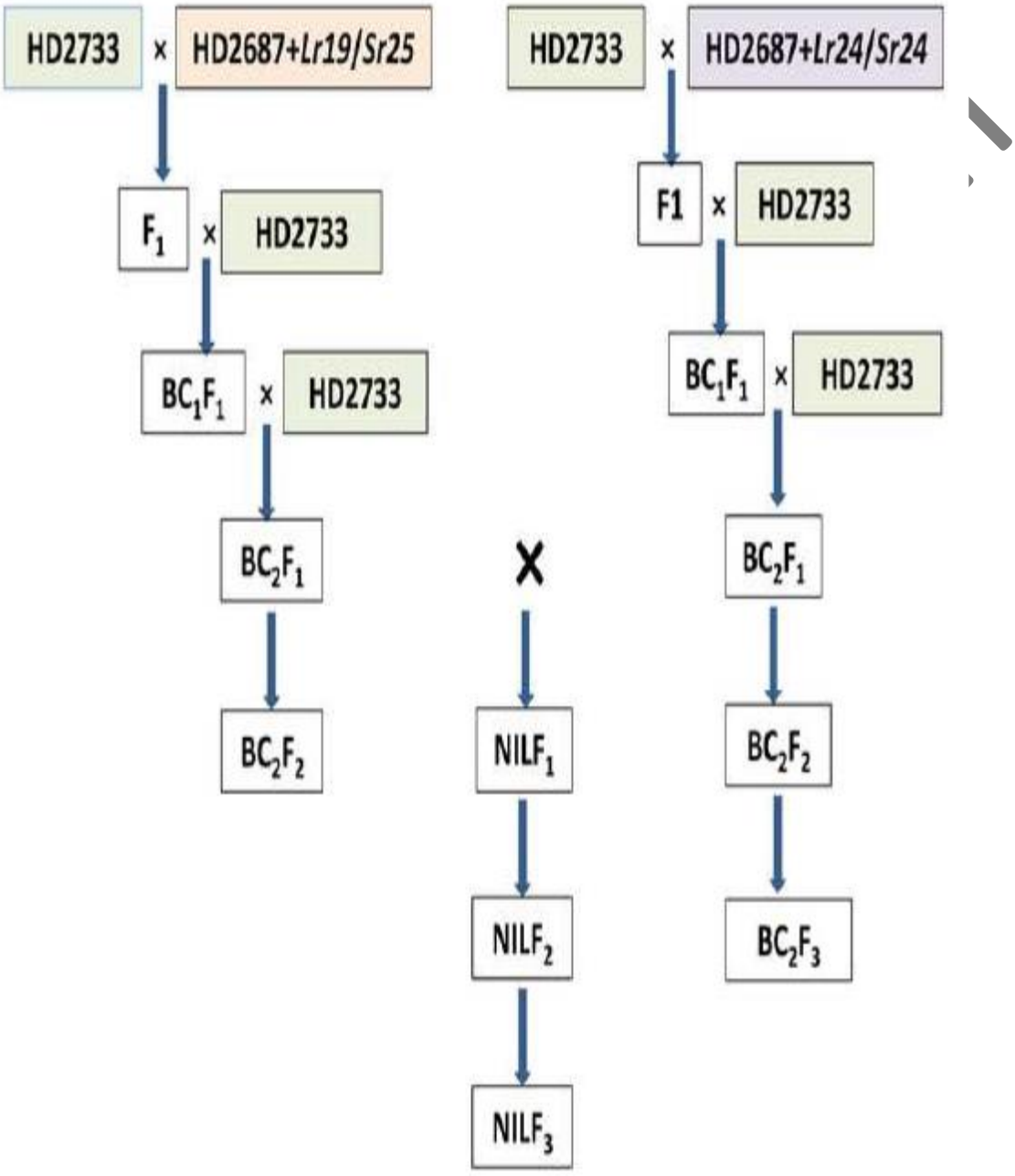
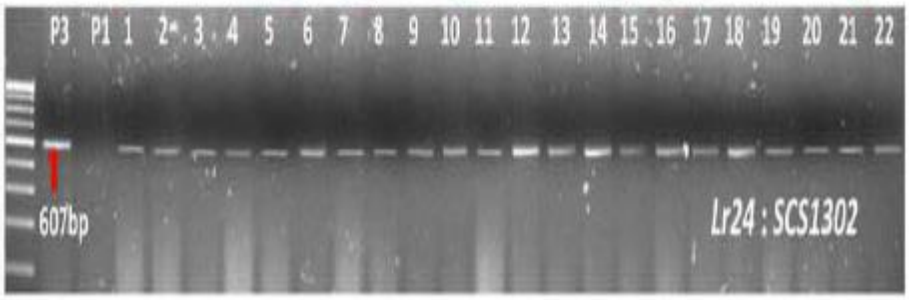


Figure 2.Foreground selection for *Lr19+Lr24* positive plants homozygous for both genes

Foreground selection for *Lr19* + *Lr24* positive plants in $NILF_2$ generation

★ : Plants homozygous for target gene

Screening for non segregating (Homozygous for *Lr24*) progeny lines in $NILF_3$



P1: HD2733 **P2:** HD2687+ *Lr19* **P3:** HD2687+*Lr24*